Bioanalytical Studies on the Effects of Chemical Compounds on the Respiratory Chain of *Candida albicans* focusing on Electrochemical Methods

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina

zu Braunschweig

zur Erlangung des Grades eines

Doktor der Naturwissenschaften

(Dr. rer. nat.)

genehmigte

Dissertation

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aus Etlidem; Ägypten

Referentin:	Professor Dr. Ursula Bilitewski
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eingereicht am:	22.08.2011
mündliche Prüfung (Disputation) am:	04.10.2011

Druckjahr	2011

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch die Mentorin der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Publications:

- Hassan R.Y.A. and Bilitewski U., (2011) A viability assay for *Candida albicans* based on the electron transfer mediator 2, 6 -Dichlorophenolindophenol (DCIP); *Analytical biochemistry*.
- Wesolowski J.*, Hassan R.Y.A.*, Reinhardt K., Hodde S. and Bilitewski U., (2010), Antifungal compounds redirect metabolic pathways in yeasts: metabolites as indicators of modes of action; *Applied Microbiology*.
- Wesolowski J., Hassan R.Y.A., Hodde S., Christian B. ,Bilitewski U., (2008), Sensing of oxygen in microtiter plates: a novel tool for screening drugs against pathogenic yeasts, *Analytical and Bioanalytical Chemistry*.
- ***Authors have an equal contribution**

Conferences

- Hassan RY. A., Buschart A. and Bilitewski U. (2010), Direct Electron Transfer from Viable Yeast cells to Electrodes; 61st Annual Meeting of the International Society of Electrochemistry, Nice-France (*Oral Presentation).*
- Hassan R.Y. A., Bilitewski U. (2010), A Novel Electrochemical Method to Determine the Viability of Pathogenic Yeasts (*Candida albicans*), Annual Meeting of the German Society of Hygiene and Microbiology (DGHM) and the Association for General and Applied Microbiology (VAAM), Hannover-Germany (*Poster*).
- Hassan R.Y.A and Bilitewski U., (2009), Direct communication between living yeasts, in particular *Candida albicans*, and Electrode, Annual Meeting of the German Society of Hygiene and Microbiology (DGHM), Hannover- Germany (*Oral Presentation*).
- Cui S., Klippel N., Hassan R.Y.A. and Bilitewski U., (2009), Treatment of Candida albicans with genistein enhances macrophage responses, Annual Meeting of the German Society of Hygiene and Microbiology (DGHM), Hannover- Germany (*Poster*).

Acknowledgements

This PhD thesis is definitely the most significant scientific accomplishment in my life and it would be difficult to be completed without people who supported and helped me. Therefore, it is a great pleasure to show here my appreciation to those people.

In the beginning, I've to admit that the switching from chemistry (my main major) to biology (current interest) is difficult. But to have a good supervisor this makes it easy. Therefore, it is very hard to overstate my gratitude to my PhD supervisor, Professor Dr. Ursula Bilitewski. With her enthusiasm, her motivation, and her great efforts to explain things clearly and simply, really she helped me too much to make everything possible. During my thesis-time, she provided a strong encouragement, good teaching and good working atmosphere. I am also very happy because she gave me the chance to present several oral and poster presentations, in local and international meetings; which provided me good experiences. Again thank you Ursula for being a patient and wonderful supervisor.

My sincere and deepest gratitude to Prof. Dr. Uwe Schröder, this is not only for reviewing this dissertation but also for sharing ideas in the scientific discussion of my thesis committee meetings in the last three years.

My deepest thanks to Prof. Dr. Ludger Beerhues for agreeing to be the chairman of my PhD defense.

My special thanks to Prof. Dr. Peter Müller for sharing his thought in the scientific discussion during my thesis committee meetings

My thanks and appreciation goes out to all my colleagues, both past and present, at BiSA group, my former colleagues (Bianca Lüderitz, Janine Wesolowski, Nina Klippel, Shuna Cui, Dörthe Sokolis and Rene Wetzel), and currently colleagues (Anna Buschart, Katja Gremmer, Daniela Evers, Karin Plank-Schumacher, Mohammed El-Mowafy, Hani Kaba, Carolin Lewark, Fardous El-Senduny and Iska Schimpf), they are not only great colleagues but they are also dear friends. I'll always remember the long working days in the lab, our Monday group meetings, birthday's parties, and Betriebsausflügen. I am very lucky to have such supportive family members on my side.

Anna Buschart requires a special mention for being such an excellent collaborator and a good friend. She is an amiable and reliable person to work with; I think we learned some valuable information from the SLN1 work. I'm very much appreciated with her enthusiasm, willingness to do further experiments, and amazing ability to understand the new ideas.

Thanks to Dr. Florenz Sasse and Mrs. Bettina Hinkelmann for providing me the mutants (COXs and FREs) of *S. cerevisiae.*

My deep thanks to Dr. Heinrich Steinmetz for tryptophol analysis by the LC-MS.

My sincere thanks to all members of applied organic chemistry dept. at the Egyptian National Research Center (NRC), and my special thanks to Prof. Dr. Hassan Naguib for his continued encouragement and friendly support.

To all my Egyptian friends, thanks for keeping in touch with me during my stay in Germany.

I wish to express my deep sense of gratitude to the Egyptian ministry of higher education and scientific research, the Egyptian National Research Centre and Helmholtz Centre for Infection Research for the financial support and for all help and the possibilities during this work.

To my parents, I would like to say that: No amount of gratitude would show the appreciation I have for you and for everything you have done for me. To my brothers, and sister, big hug for being there for me.

Very special thanks to my wife for always being there, encouraging, helping, providing a loving environment and taking care of me and my children (Youssef and Mahmoud). I am so excited to return back to our *new Egypt* and start the next phase of our life together with our children.

To all those who are not mentioned here, maybe due to lack of my memory power, my deepest apologies and too much thanks.

Rabeay Younes Abdelfatah Hassan!

Braunschweig, Germany

22/08/2011

Thesis organization

This thesis ***''Bioanalytical Studies on the Effects of Chemical Compounds on the Respiratory Chain of Candida albicans focusing on Electrochemical Methods''*** presents the results of my PhD project carried out at the laboratory of Biological Systems Analysis group (BiSA), department of Chemical Biology (CBIO), Helmholtz Centre for Infection Research and under the supervision of Professor Dr. Ursula Bilitewski.

This dissertation consists of four interrelated chapters.

In the first chapter, the problem definition and the work motivation (the main objective) was explained in details, combined with a general introduction to the human fungal pathogen *Candida albicans*, and the full description of its respiratory chain. Although *C. albicans* was the target organism through this study, another pathogenic organism (*Candida glabrata)* and non-pathogenic organism (*Saccharomyces cerevisiae*) were used to elucidate the reaction mechanisms.

In the second chapter, a new strategy for understanding the mode of action of the respiratory chain inhibitors was designed. In this strategy, several biochemical assays were implemented for determination of oxygen uptake, ROS formation, cell viability and metabolites analysis such as ethanol and glycerol production.

In the third and the fourth chapters, two bio-electrochemical approaches were optimized to serve as new tools for identification of the electron transfer activity. In the third chapter, the self-mediated electron transfer system was used to evaluate the performance of the classical respiratory pathway; the decrease in the electrical signal is proved to be an indication of the defect in the classical respiratory pathway. In the fourth chapter, the DCIP-mediated electron transfer system was exploited to identify the activity of the respiratory chain complex I (NADH-dehydrogenase). Moreover, by the use of this bio-electrochemical approach we were able to distinguish between the complex-I-positive and negative organisms (e.g. differentiation between *C. albicans* and *S. cerevisiae*). We also report that in *C. albicans*, Sln1 osmo-sensor histidine kinase acts with the respiratory systems in particular at the respiratory chain complex I.

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LIST OF SCHEMES

AA	Antimycin A
ADH	Alcohol-dehydrogenase
ADP	Adenosindiphosphate
AOX	Alternative oxidative pathway
ATP	Adenosintriphosphate
C. albicans	*Candida albicans*
Complex I; C(I)	Mitochondrial complex I
Complex II; C(II)	Mitochondrial complex II
Complex III; C(III)	Mitochondrial complex III
Complex IV; C(IV)	Mitochondrial complex IV
CoQ	Coenzyme Q (ubiquinone)
COX	Cytochrome oxidase
CPE	Carbon Paste Electrode
CRE	Classical respiratory Chain
CV	Cyclic Voltammetry
DCIP	Dichlorophenolindophenol
DMSO	Dimethyl sulfoxide
ETC	Electron Transport Chain
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GDH	Glycerol-dehydrogenase
HKs	Histidine Kinases
LSV	Linear Sweep Voltammetry
MAP	Mitogen activated protein
MAP-kinase	Mitogen-activated protein kinase
MFCs	Microbial Fuel cells

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
Myxo	Myxothiazol
NADH	Nicotinamide adenine dinucleotide
OD	Optical Density
PAR	parallel respiratory pathway
PBS	Phosphate buffered saline
Pyrrol	Pyrrolnitrin
ROS	Reactive oxygen species
Rot	Rotenone
S. cerevisiae	*Saccharomyces cerevisiae*
SHA	Salicylhydroxamic acid
TCA	Tricarboxilic acid
TTFA	Thenoyltrifluoroacetone
WST1	4-[3-(4-Jodophenyl)-2-(4-Nitrophenyl) -2H-5-Tetrazolio]-1,3-Benzoldisulfonate
YPD	Yeast extract peptone dextrose

Abstract

Candida albicans is the most prevalent human fungal pathogen. This fungus colonizes mucosa and skin in approximately 50% of healthy individuals. However, *C. albicans* causes opportunistic infections in immunocompromised patients that range from superficial to systemic infection. Therefore, new antifungal agents are urgently needed. The electron transport chain plays a key role in processes such as oxidative phosphorylation, aerobic metabolism, and in the expression of virulence factors of *C. albicans*. Thus, the respiratory chain can be a potential drug target. The electron transport chain of *C. albicans* comprises three respiratory pathways, namely the classical, alternative oxidative and parallel pathway. In order to evaluate the respiratory chain status, the complexity of this structure necessities sensitive and mechanistic assays.

The work presented in this study demonstrates the use of biochemical and electrochemical assays to investigate these respiratory pathways. By applying biochemical methods, the activity of the respiratory chain and the importance of specific complexes were elucidated. For example, growth inhibition tests showed the importance of the inhibited complexes for growth in different media. Disruption of the respiratory pathway at defined points, e.g. by rotenone, increased the rate of oxygen uptake. Respiratory deficiency increased the sensitivity of *C. albicans* to oxidative stress induced by complex I and III inhibitors. Analysis of metabolites, determination of ethanol and glycerol, exhibited the role of the fermentation pathway in the cell survival.

Two electrochemical approaches were applied in this study:

(a) A self-mediated electron transfer approach was utilized to investigate the classical respiratory chain, in particular the activity of the respiratory chain complex IV. By this technique the effect of complex IV inhibitors on the respiratory activity can be easily detected.

(b) DCIP-mediated electron transfer approach was used to evaluate the activity of NADH-dehydrogenase complex I in particular. This approach can be used to discriminate between the different organisms.

Zusammenfassung

Candida albicans ist der häufigste humanpathogene Pilz. Er besiedelt die Haut und Schleimhäute von ungefähr 50% aller gesunden Individuen. Andererseits löst *C. albicans* bei Störungen des Immunsystems Infektionen aus, die oberflächlich oder systemisch sein können. Daher werden neue Antimykotika dringend gebraucht. Die Elektronentransportkette nimmt eine Schlüsselposition in Prozessen wie der oxidativen Phosphorylierung, dem aeroben Stoffwechsel und der Expression von Virulenzfaktoren von *C. albicans* ein. Daher kann die Atmungskette ein potenzielles Ziel für Wirkstoffe sein. Die Elektronentransportkette von *C. albicans* umfasst drei Wege, den klassischen, den alternativen oxidativen und den parallelen Weg. Um den Zustand der Atmungskette zu bewerten, sind aufgrund der Komplexizität dieser Struktur empfindliche und mechanistische Untersuchungen notwendig.

In dieser Arbeit wurde der Nutzen biochemischer und elektrochemischer Untersuchungsmethoden für diese Atmungswege demonstriert. Durch die Anwendung biochemischer Methoden wurden die Aktivität der Atmungskette und die Bedeutung spezifischer Komplexe beleuchtet. Zum Beispiel zeigten Untersuchungen zur Wachstumshemmung die Bedeutung der gehemmten Komplexe für das Wachstum in verschiedenen Medien. An definierten Punkten führten Unterbrechungen der Atmungskette, z. B. durch Rotenone, zu einer Steigerung der Sauerstoffaufnahme. Atmungsdefekte erhöhten die Empfindlichkeit von *C. albicans* für oxidativen Stress, der durch Inhibitoren von Komplex I und III induziert wurde. Die Analyse von Metaboliten durch die Bestimmung von Ethanol und Glycerin stellte die Funktion der Gärung im Überleben der Zellen heraus.

Zwei elektrochemische Ansätze wurden in dieser Arbeit verfolgt:

a) Ein Ansatz über selbst-vermittelten Elektronentransfer wurde benutzt um die klassische Atmungskette zu untersuchen, insbesondere die Aktivität von Komplex IV. Durch diese Methode kann die Wirkung von Inhibitoren von Komplex IV auf die Atmungsaktivität einfach detektiert werden.

b) Ein Ansatz mit DCIP-vermitteltem Elektronentransfer wurde verwendet um speziell die Aktivität des NADH-Dehydrogenase-Komplex I zu untersuchen. Dieser Ansatz kann verwendet werden um zwischen verschiedenen Organismen zu unterscheiden.

Motivation and aim of the thesis

Candida albicans is the most frequent human fungal pathogen. It is closely related to the non-pathogenic yeast *Saccharomyces cerevisiae*. Differences between both organisms, however, are found not only in regulatory mechanisms of signal proteins or signal transduction pathways, but also in fundamental structures such as the respiratory chain and the morphology. The respiratory chain of *C. albicans* is more branched than that of *S. cerevisiae* and *C. albicans* can switch between yeast and hyphal forms. Both properties contribute to the virulence of *C. albicans*.

The complex and flexible structure of the electron transport system in *C. albicans* allows the organism to adapt to the inhibition of single complexes or pathways of the respiratory chain by the redirection of metabolic pathways or the expression of additional genes. Thus, the aim of this thesis was the establishment of a set of bioanalytical assays, the combination of which allows the elucidation of modes of action of chemical compounds and the relevance of distinct protein complexes. Electrochemical detection methods were of particular interest, because they offer the possibility of direct communication with the cellular electron transfer system.

Practical considerations to achieve the objectives of the study:

In order to achieve the main goal of this study, two experimental classes were used, biochemical and electrochemical methods (Figure 1). The following tasks were performed.

- Optimization of a sensitive biochemical assay to detect the effect of electron transport chain-inhibitors on the oxygen uptake of yeasts.
- The use of the yeast metabolite profiles as a predictor of the mode of action of the respiratory inhibitors.
- Design of a new strategy to identify the mode of action of respiratory chain inhibitors.
- Establishment of bio-electrochemical systems to:
    - Detect the viability of *C. albicans* via measuring the electron transfer activity from the viable yeast cells to the electrode system.
    - Measure the activity of NADH-dehydrogenase (complex I) and discriminate between complex I-negative and positive organisms.
    - Study the function of non-mitochondrial proteins (histidine kinases) for the respiratory activity of *C. albicans.*

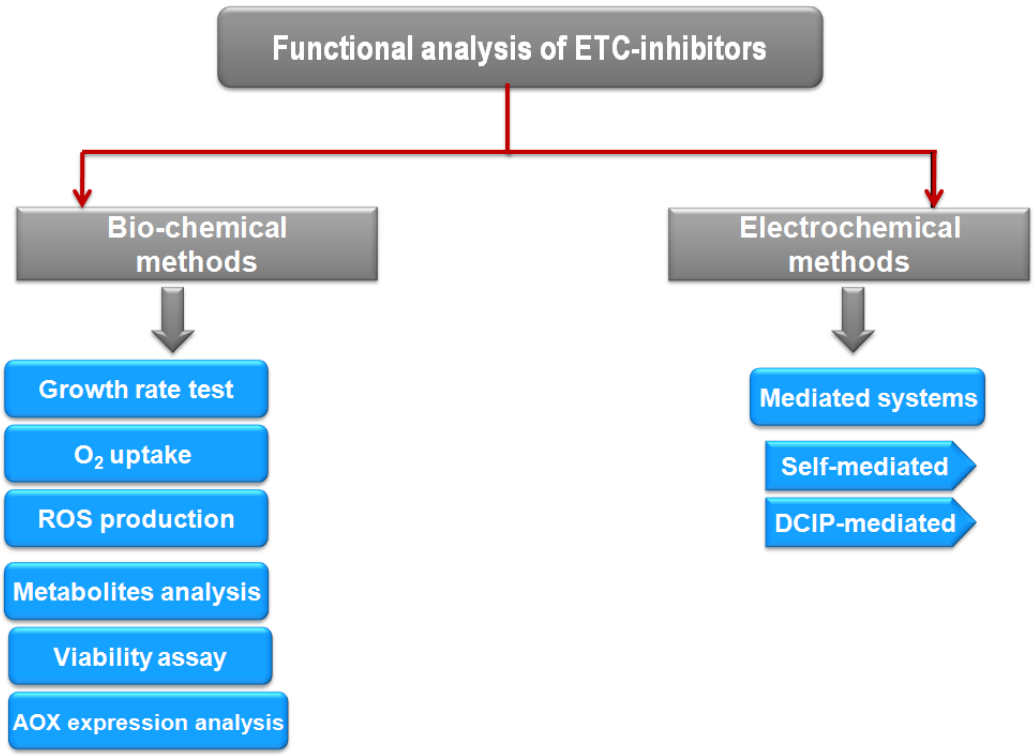

Figure 1: Experimental setup of the study which includes biochemical detection methods and bioelectrochemical methods.

Background and Overview of the study

1. *Candida albicans*

The fungi constitute a highly diverse and successful group of organisms that include well-known species such as yeasts, molds and mushrooms, and are classified as a biological kingdom of eukaryotic organisms distinct from animals and plants. Most fungi are unnoticed because of their small size, but there are some fungi that have made a great impact in human history; for example *Saccharomyces* sp. that are used worldwide in the production of alcoholic products and in bread making, or fungi (e.g. *Penicillium* sp., *Acremonium* sp. and *Aspergillus* sp.) that are used for the production of antibiotics and enzymes. However, there are some fungi (e.g. *Candida* sp.) that cause human diseases. Regardless of their classification, several fungi can proliferate in two distinct forms, i.e. filamentous growth and yeast growth. Filamentous fungi grow as tubular elongated cells called hyphae. Yeast-like fungi grow as single cells, where new cells separate or bud from the mother cell. Most fungi are limited to one of these forms of growth, but some fungi are able to switch between yeast and hyphal growth. These fungi are termed dimorphic [1-3].

Fungal pathogens can be divided into two general classes, primary pathogens and opportunistic pathogens. Fungi in the former class usually have an environmental reservoir and infect individuals who have either been exposed to a large antibiotic dose or who are immunologically naive to the fungus. Opportunistic pathogens take the advantage of debilitated or immunocompromised hosts to cause infection [2, 4].

The genus *Candida* includes nearly 200 species, some of them (e.g. *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei* and *C. guilliermondii*) are associated with human or animal infections, of which *C. albicans* is the major fungal pathogen of humans. *C. albicans* like most *Candida* species (except *C. glabrata*) is a diploid fungus with dimorphic morphology, as it can switch from yeast to hyphal form, and vice versa, in dependence of environmental factors [5]. *Candida* infections are a serious problem, especially in individuals whose immune defense mechanisms have been weakened [6, 7]. *C. albicans* can colonize skin and mucosal surfaces of healthy people and thus occurs commensally in the gastrointestinal tract, oral cavity and vagina. However, it can cause superficial infections [8]. But it also can enter the bloodstream by direct penetration from the epithelium after tissue damage, or by dissemination from biofilms formed on medical devices introduced into the patient, e.g. catheters, dental implants, or artificial joints [9]. Then yeast cells disseminate

with the blood flow and infect almost all inner organs, including lungs, kidney, heart and liver.

1.1. Susceptibility of C. albicans to antifungal agents

Fungal infections are a common issue of both medical and agricultural communities, and both struggle with the evolution of pathogen resistance to antimicrobial agents so that continuous efforts are required to discover new compounds with appropriate biological activities [2, 10]. The mode of action of antifungal compounds can be classified according to the targeted pathways. For example following groups can be distinguished: (i) inhibitors of nucleic acid metabolism (e.g. by flucytosine) (ii) inhibitors of protein synthesis, (iii) effectors of the cell membrane structure (e.g. by polyenes or by inhibition of ergosterol biosynthesis by azoles), (iv) inhibitors of cell wall composition (e.g. via inhibition of ß-glucan biosynthesis by echinocandins) and (v) inhibitors of central metabolic pathways (e.g. carbon or energy metabolism affected by electron transport chain inhibitors) [10-12].

1.2. Virulence (Pathogenesis) factors of C. albicans

Microorganisms which cause disease have special factors which are nominated as virulence factors. These factors contribute to the pathogenicity of the microorganisms and to their survival in the hostile environment within the body of their host. There are many factors that contribute to the pathogenicity of *C. albicans*. These include the secretion of hydrolytic enzymes (such as secretory aspartyl proteinases and phospholipases), adhesion to host cells and tissues, dimorphic transition (yeast-to-hypha), biofilm formation and the immune status of the host. Various signals control the expression of the virulence factors. Oxygen availability, temperature, concentration of ions, and pH are some of the known signals that change the microbial virulence. In the next section more details will be given for each of the virulence factors.

1.2.1. Morphological changes

Most dimorphic fungi that are human pathogens grow as filaments in their external habitat but grow as budding yeast into diseased tissues, e.g. *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Blastomyces dermatitidis* [13]. In contrast, the hyphal growth form of *C. albicans* is important for virulence because the ability to switch between yeast, hyphal and pseudohyphal morphologies (Figure 2) is often considered to be necessary for virulence [14].

Hyphae and pseudohyphae are able to grow invasively into agar, and one theory suggests the hyphal and pseudohyphal forms would promote the invasive growth into tissues during the early infection and also colonization of inner organs. When the cultivation process takes place at 30 °C, *C. albicans* grows as unicellular budding yeast (similar to diploid *Saccharomyces cerevisiae*). However, the phenotypic switching to hyphal growth is usually induced by growth at 37 °C and neutral pH (~7.0) with the addition of external stimuli such as serum, N-acetylglucosamine [15, 16] or glucose limitation [17, 18].

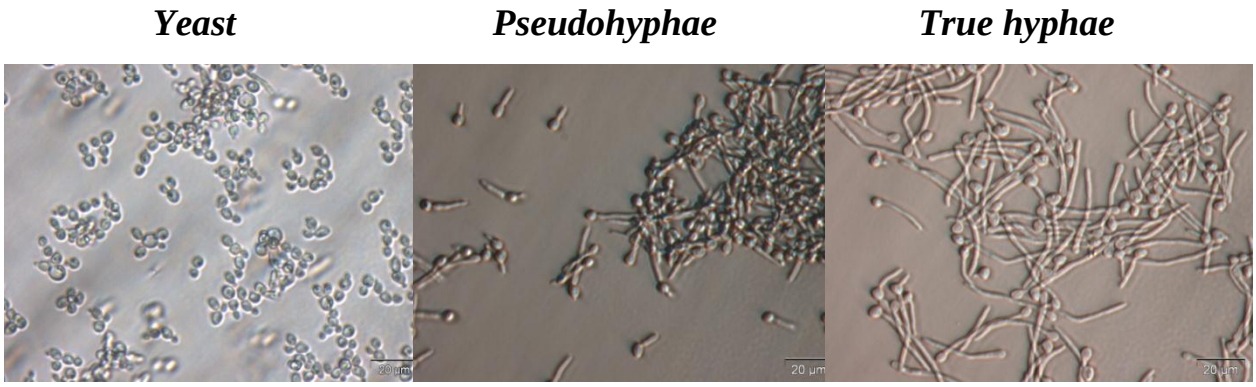

Figure 2: Yeast, pseudohyphal and true hyphal morphologies of *C. albicans*. The yeast form of *C. albicans* is similar in the appearance to the *S. cerevisiae* cells.

1.2.2. Biofilm formation

C. albicans is able to form a heterogeneous architecture called biofilm (Figure 3), which consists of a mixture of yeast and filamentous cells enveloped by a matrix of polysaccharides and proteins. The ability to adhere to surfaces (inert or living) and develop as a multicellular community is an adaptation used by most microorganisms to survive in changing environments. The capability of *C. albicans* to form biofilms is an important mechanism for persistence, facilitating its growth on different tissues and a broad range of abiotic surfaces used in medical devices [19]. Mature biofilms are characterized by the production of a thick extracellular matrix and an altered resistance phenotype to common antifungal agents [19]. In addition, the biofilm structure is highly resistant to the immune system, and is usually involved in chronic infections of *C. albicans* [20].

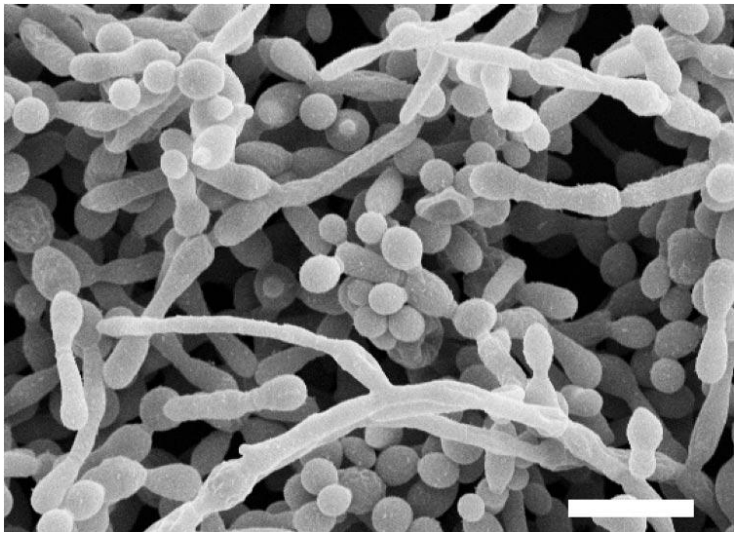

Figure 3: Scanning Electron Microscopy image of a mature (48 h) *C. albicans* biofilm. *C. albicans* forms a dense biofilm composed of yeast cells, hyphae, and extracellular matrix [21].

1.2.3. Adhesion

The cell wall of *C. albicans* is comprised primarily of the polysaccharides mannan, glucan, and chitin. Mannans represent about 23%, glucans 40-60%, proteins 6-25%, lipids 1-7%, and chitins 0.6-9% of the dry weight of the cell wall. Ultrastructural studies of the cell wall have indicated a complex microarchitecture of variable thickness, which is composed of several layers [22]. It is generally agreed that mannan is distributed throughout the cell wall, whereas the inner cell wall is composed mainly of chitin and glucan. The outer layer of the cell wall is consisted of mannan or mannoprotein, which has been described as a mucous coat or capsule, and which is shed during infection.

Adhesion of *C. albicans* to host tissue (Figure 4) is an important virulence factor in the development of disease. Poorly adherent strains of *C. albicans* were less virulent in animal models [23]. Invasive strains of *C. albicans* recovered from patients show better adherence than strains recovered from asymptomatic carriers. The attachment is believed to be due to a specific interaction of *C. albicans* cell wall components with host cell surfaces [24], In *C. albicans* mannoprotein, glucan, chitin, cell wall proteins, and lipids are possible adhesions, while, the macromolecule of the mannoprotein is a strong adhesion [25].

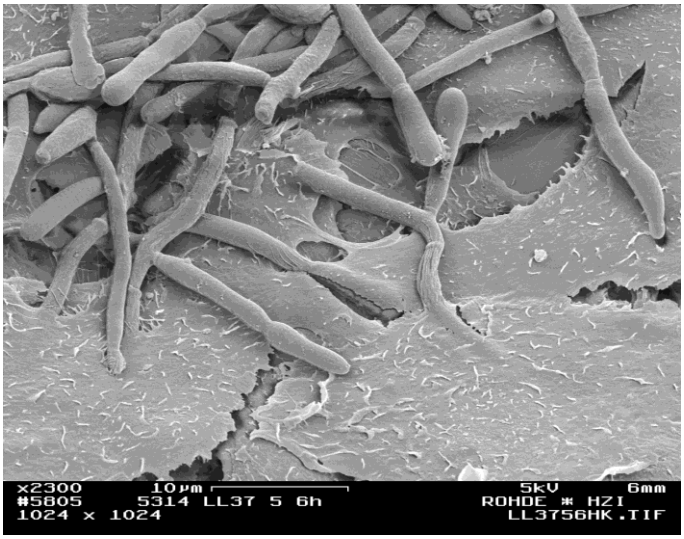

Figure 4: The electron microscopic picture shows the ability of the hyphal form of *C. albicans* to adhere and penetrate into the epithelial cell (Daniela Evers, BiSA, HZI).

1.2.4. Exoenzymes production

To aid in the invasion of host tissues, microbial cells possess constitutive and inducible hydrolytic enzymes that destroy or derange constituents of host cell membranes, leading to membrane dysfunction and/or physical disruption [26, 27]. Since membranes are made up of lipids and proteins, these biochemicals constitute the target of enzyme attack. *C. albicans* secretes enzymes which are considered to be essential for their pathogenesis; these are categorized into two main types, proteinases, which hydrolyze peptide bonds, and phospholipases, which hydrolyze phospholipids [28, 29].

1.3. The Impacts of respiratory chain on the virulence factors of C. albicans

Initially, mitochondria were considered as the house of power for the living system that supplies the cells with the needed energy. Currently, the mitochondria are well known as being essential to many aspects of the biology and virulence activity of microorganisms [30]. The degree of involvement of respiratory chains in the regulation of the virulence factors of *C. albicans* will be illustrated in the next section.

1.3.1. Hyphae formation

It was mentioned before that the morphogenesis is an important parameter of the virulence of *C. albicans*. Blocking the morphological changes decreases the pathogenicity of this fungus.

A number of studies exhibited an interesting relationship between the respiratory pathways and morphological change (yeast-to-hyphae transition) in *C. albicans*, as shown below:

- The hyphal formation of *C. albicans* can be suppressed by blocking the enzymatic functions of the respiratory chains [31, 32].
- Specific knock-out of genes involved in the functions of mitochondrial-complex I, (*NDH51*), resulted in a filamentation defect even at low concentrations of glucose [33].
- The mutant of *C. albicans*, Δ*goa1* (Growth and Oxidant Adaptation), which has a defect in the mitochondrial complex I led to a defect in the hyphal formation and exhibited avirulence behavior in the systemic model of candidiasis, could be due to the increase in the sensitivity to oxidants and neutrophils killing [34].

1.3.2. Biofilm formation

As was explained before, the biofilm formation carries negative clinical implications, because cells within the biofilm are protected from host immune responses and from the action of antifungals. Interestingly, blocking the respiratory chain leads to prevention of biofilm formation in *C. albicans* [35]. The increase of ROS production (due to the blocking of specific sites of respiratory chains) may play a role in destruction of biofilms [36].

1.3.3. Cell wall biogenesis

The yeast cell wall is essential for cell integrity and interactions with the environment. In *C. albicans*, the cell wall governs key functions including adherence and pathogenicity, and hence serves as a pathogen-specific antifungal drug target [37]. It has been recently demonstrated that defects in the cell wall integrity of *C. albicans* are linked to the efficacy of respiratory chains, and it was indicated that the mitochondrial function plays a role in the fungal cell wall biogenesis [38].

1.3.4. Adhesion of C. albicans

Adhesion of *C. albicans* to host cells represents a virulence factor and a significant step in the development of candidiasis. It has been shown that a deficiency in the respiratory system led to a reduction in the adhesion properties of *C. albicans* to the host cells [39].

1.3.5. Exoenzymes production

As was mentioned previously, in order to accelerate and facilitate its invasion through the host cell, *C. albicans* secretes specific enzymes which are considered to be integral to their pathogenesis [28, 29]. The decrease of the efficacy of mitochondrial respiratory chains causes an inhibition of the protease and phospholipase production by *C. albicans* [40].

The linkage of virulence factors and the activity of the respiratory chains in *C. albicans* was clarified in the previous section (1.3) and is summarized in Figure 5.

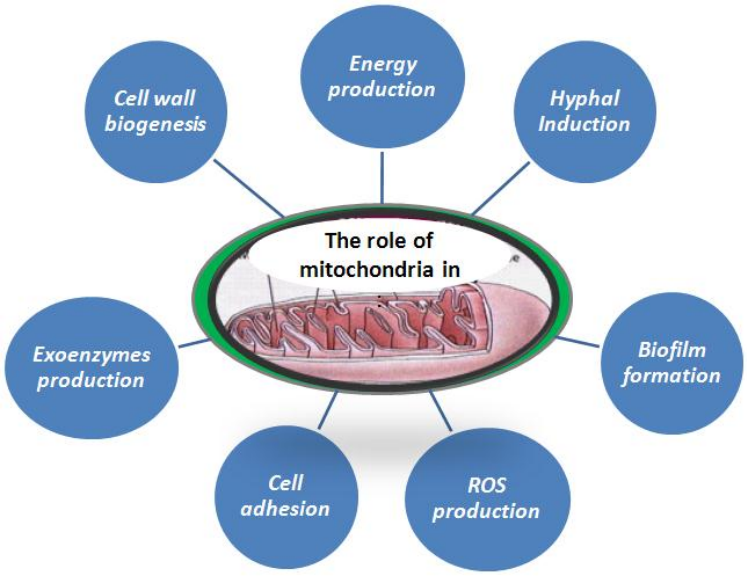

Figure 5: The importance of respiratory chain to the virulence factors of *C. albicans*.

2. Energy Metabolism

Pyruvate produced by glycolysis is the central precursor of generating energy. The two possible pathways for pyruvate can be classified into respiration and fermentation. Yeasts can employ two major strategies for energy production during the growth on glucose: respiration or fermentation. Both respiration and fermentation employ glycolysis as the central pathway, but respiration is energetically more efficient than fermentation. Most eukaryotes are obligate or facultative aerobes and therefore predominantly employ respiration in the presence of oxygen, while the fermentation pathway is used only in the absence of oxygen. However, *S. cerevisiae* exhibits a unique dependence on the fermentation pathway and therefore

ferments sugars to ethanol instead of using respiration, even under aerobic conditions [41, 42].

2.1.1. Anaerobic conditions

Yeasts can be categorized in several groups according to their modes of energy production, utilizing respiration or fermentation. It is important to note that these processes are mainly regulated by environmental factors. The best documented being the availability of glucose and oxygen. Hence yeasts can adapt to varying growth environments. In yeast, pyruvate is reduced to ethanol, as an alternative energy source. This reduction is mediated by a two-step process. Pyruvate is decarboxylated to acetaldehyde by pyruvate decarboxylase in an essentially irreversible reaction. The second step, the reduction of acetaldehyde to ethanol by NADH, is catalyzed by alcohol dehydrogenase (Figure 6).

According to the stoichiometry of alcoholic fermentation two molecules of ATP are produced per molecule of glucose converted into ethanol. Additionally, the alcoholic fermentation is a redox-neutral process, since the NADH produced during the oxidation of glyceraldehyde-3-phosphate by glyceraldehyde-3-phosphate dehydrogenase is subsequently reoxidized in the reduction of acetaldehyde to ethanol by alcohol dehydrogenase [43].

2.1.2. Aerobic conditions

In aerobic organisms pyruvate is the important source of acetyl coenzyme A (acetyl-CoA), acetyl-CoA is a central metabolite in carbon and energy metabolism. Where pyruvate is oxidized (with loss of the carboxyl group as CO_2), the remaining two-carbon unit becomes activated with coenzyme A to form acetyl-coenzyme A. The Acetyl-coA is metabolized by the tricarboxylic acid cycle (fully oxidized) to yield CO_2. The electrons removed from this oxidation process are subsequently passed through the mitochondrial electron transport system and used to generate molecules of ATP by oxidative phosphorylation (Figure 6).

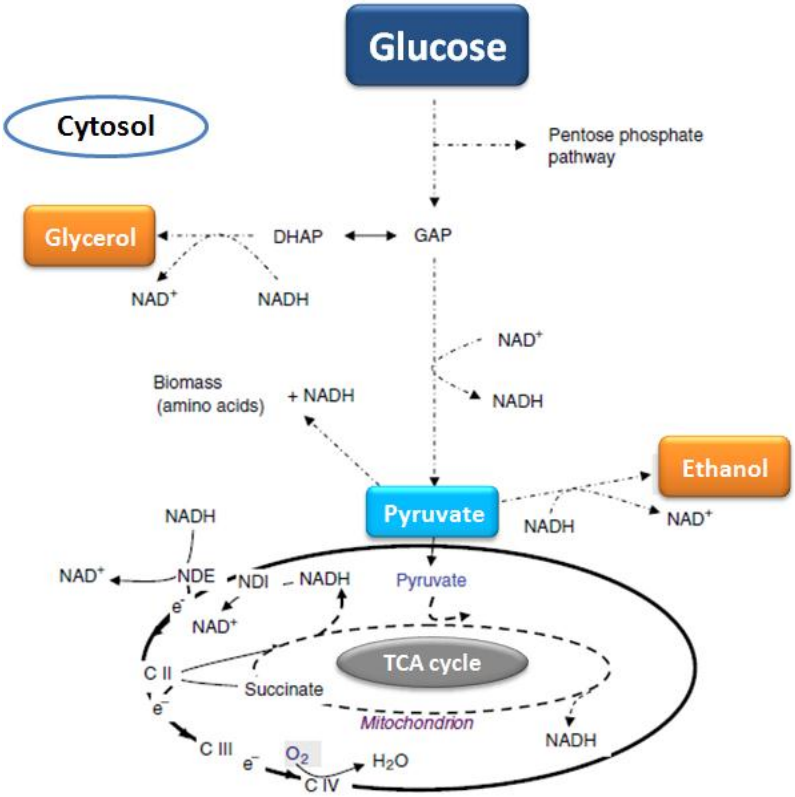

Figure 6: Simplified scheme of the metabolic pathways related to respiration, alcoholic fermentation and glycerol production.

2.1.2.1. The Tricarboxylic Acid Cycle (TCA cycle)

In eukaryotic cells, glycolysis occurs in the cytoplasm, while the TCA cycle reactions and all subsequent steps of aerobic metabolism take place in the mitochondria. Pyruvate must first enter the mitochondria to enter the TCA cycle. The oxidative decarboxylation of pyruvate to acetyl-CoA is the connecting link between glycolysis and the TCA cycle. The pyruvate dehydrogenase complex (PDC) is a non-covalent assembly of three different enzymes (E1 (2-oxo acid dehydrogenase), E2 (acyltransferase), and E3 (lipoamide dehydrogenase)) operating in concert to catalyze successive steps in the conversion of pyruvate to acetyl-CoA. The active sites of all three enzymes are not far from one to another, and the product of the first enzyme is passed directly to the second enzyme and so on, without diffusion of substrates and products through the solution [44, 45]. Active-site coupling is used to catalyze decarboxylation of pyruvate (E1), esterification of aldehydes to CoA (E2), and reduction of NAD^+ to NADH (E3) [45, 46].

2.1.2.2. Functioning of electron transport chain

In eukaryotic cells, mitochondria carry out the final steps of the respiration process and generate the bulk of ATP through oxidative phosphorylation to release CO_2 and reduce oxygen to water. The acetyl-CoA enters the TCA cycle to generate NADH + H^+ (Figure 7). Fatty acids are oxidized entirely within the mitochondrion by beta-oxidation, generating mitochondrial acetyl-CoA, NADH + H^+, and $FADH_2$. Two electrons are transferred from NADH +H^+ to NADH dehydrogenase (complex I) or from $FADH_2$-containing enzymes such as the electron transfer factor dehydrogenase or succinate dehydrogenase (complex II) to reduce ubiquinone [coenzyme Q (CoQ)] to ubisemiquinone, and then to ubiquinol. Subsequently, the electrons from ubiquinol are transferred through the electron transport chain (ETC) successively to complex III (*bc1* complex), cytochrome *c*, complex IV (cytochrome *c* oxidase), and finally to oxygen (1/2 O_2) to yield H_2O. The energy that is released as the electrons flow down the ETC is used to pump protons out across the mitochondrial inner membrane through complexes I, III, and IV. This creates a proton electrochemical gradient [47, 48]. The energy to convert ADP + P_i to ATP comes from the flow of protons through the ATP synthetase (complex V) back into the matrix.

3. Structure of electron transport chain in yeast

ATP production is coupled to the electron transport chain in the inner mitochondrial membrane through the electron transport chain. However, the structures of electron transport chain of the chosen yeasts in the study (*C. albicans*, *C. glabrata* and *S. cerevisiae*) differ. Therefore, in this section, the main differences between the respiratory chain structures of these organisms will be given in details.

3.1. Electron transport chain of C. albicans

C. albicans is a Crabtree-negative organism and obtaining its energy relies mainly upon the (aerobic respiration) oxidation of substrates via the mitochondrial tricarboxylic acid cycle to generate ATP even in the presence of glucose [34]. However, switching to fermentation is also possible. Therefore, the electron transport chain of *C. albicans* is a vital unit for its survival, particularly if the growth medium contains a non-fermentable sugar (galactose). However, the electron transport chain of *C. albicans* is not linear and is composed of three respiratory pathways: the classical respiratory chain (CRC) or the linear respiratory chain, the alternative respiratory chain and the parallel electron transport chain (PAR), in addition there

are alternative NADH dehydrogenases (external and internal NADH-dehydrogenase), as shown in Figure 7.

3.1.1. The classical respiratory chain

The CRC of *C. albicans* provides the largest amount of cellular ATP because it is the major source of pumping protons across the mitochondrial inter-membrane space [49].

The classical respiration system in *C. albicans* consists of complexes I and II, coenzyme Q, complex III, cytochrome *c*, and complex IV, which provides the terminal reduction of oxygen into water (Figure 7). Electrons from NADH and $FADH_2$ are passed along the classical respiratory chain to the terminal electron acceptor O_2. This process is combined with pumping of protons (via complex I, III and IV) across the inner mitochondrial membrane, creating an electrochemical gradient. The electrochemical proton gradient enables the ATP synthase to produce ATP.

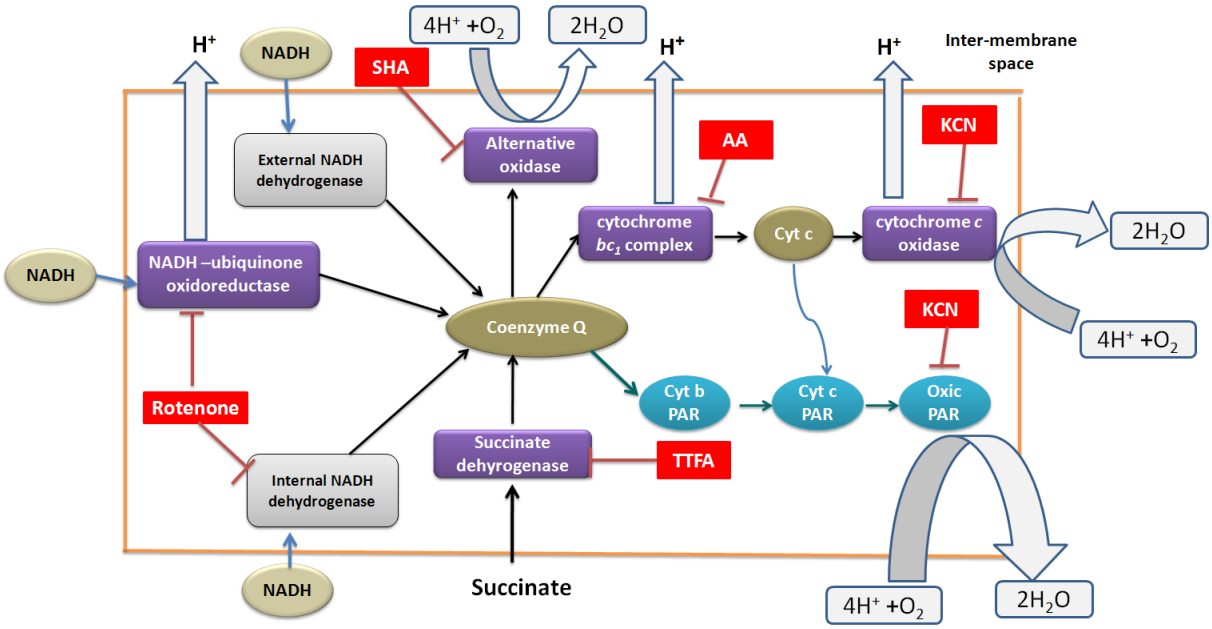

Figure 7: Schematic representation of the respiratory chain structure of *C. albicans.* NADH is oxidized by any of the NADH-dehydrogenases, in particular by the proton-pumping NADH-ubiquinone oxidoreductase (complex I), leading to reduced coenzyme Q. Coenzyme Q is also produced from the oxidation of succinate by succinate-dehydrogenase (complex II). Reduced coenzyme Q is oxidized either by oxygen through the alternative oxidase, or by cytochrome *c* in the cytochrome *bc1* complex (complex III). Cytochrome *c* is oxidized by oxygen by cytochrome *c* oxidase (complex IV). These latter reactions can

also be catalyzed by components of the parallel pathway PAR. Inhibitors are known for each of the major enzyme complexes; SHA: salicylhydroxamic acid; AA: antimycin A; KCN: cyanide; TTFA: Thenoyltrifluoroacetone.

3.1.1.1. Complex I

NADH:ubiquinone oxidoreductase (NADH-dehydrogenase) catalyzes the first step of the electron transport chain in mitochondria. It transfers electrons from NADH to a non-covalently bound FMN and then via a series of iron-sulfur clusters to the terminal acceptor, ubiquinone [50, 51]. The transfer of two electrons is coupled to the translocation of four protons across the inner membrane. Complex I in fungi is very similar to its counterpart in other forms of life [52] and its structure is an L-shaped assembly of more than 40 different proteins, seven hydrophobic components are products of the mitochondrial genome, and the remainder are nuclear gene products that are imported into the organelle [52]. Rotenone inhibits the transfer of electrons from iron-sulfur centers in complex I to ubiquinone, which leads to preventing the oxidation of NADH.

3.1.1.2. Complex II

The major component of succinate dehydrogenase (SDH) or succinate:quinone oxidoreductase is found in all aerobic organisms as a membrane-bound enzyme of the citric acid cycle. Complex II catalyzes the oxidation of succinate by ubiquinone, generating ubiquinol and fumarate. This reaction does not result in a transfer of protons across the mitochondrial inner membrane. Moreover, it is known that the catalytic function of this complex is inhibited by 2-thenoyltrifluoroacetone (TTFA) [53, 54] .

3.1.1.3. Complex III

Ubiquinol-cytochrome *c* oxidoreductase (*bc1* complex) is a component of the eukaryotic and of the bacterial respiratory chain. This enzyme catalyzes the electron transfer from ubiquinol to cytochrome *c*, which is coupled to the translocation of protons across the mitochondrial inner membrane. The yeast *bcl* complex is consisted of at least nine non-identical subunit polypeptides [55]. Only three subunits (cytochrome *b*, cytochrome *cl*, and the Fe-S protein) have known electron transfer functions. It is thought that cytochrome *b* is a trans-membrane protein in which two hemes form a trans-membrane electron circuit between two ubiquinone redox sites responsible for proton uptake and proton release across the inner-mitochondrial

membrane. The catalytic functions of complex III were inhibited by blocking one of the two pathways of cytochrome *b* oxidoreduction at either the Q_i or Q_o site (Figure 8). For example, antimycin was used to block oxidation-reduction at the Q_i site and cause changes in the optical properties of Q_i, while, myxothiazol appears to block the oxidation-reduction at the Q_o site [55].

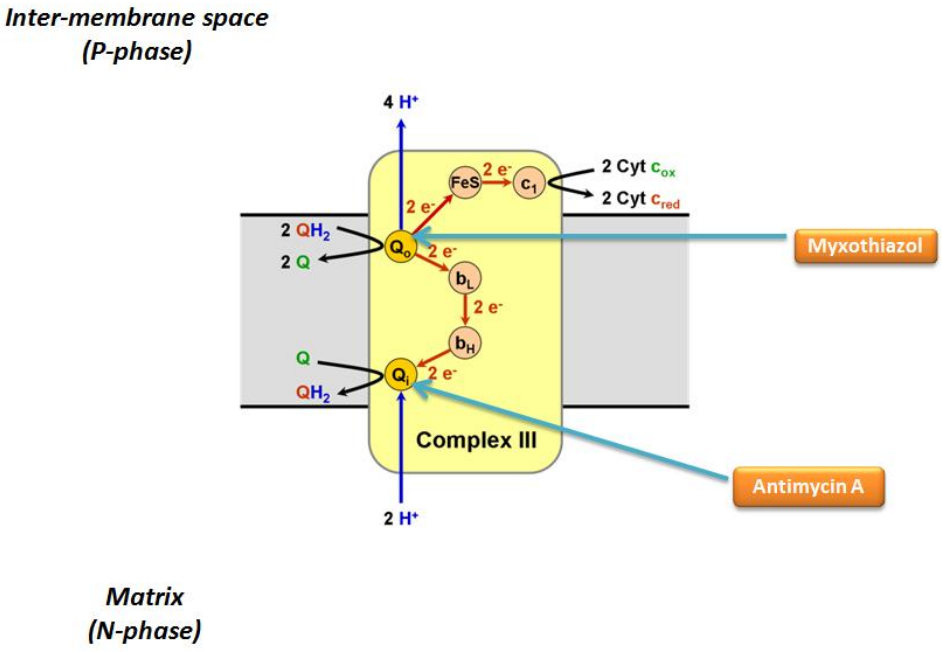

Figure 8: The scheme shows the branched, cyclic pathway of electron transfer through the redox centers of the cytochrome *bc*1 complex. The figure shows the sites at which antimycin and myxothiazol inhibition sites within the complex III [56].

3.1.1.4. Complex IV

Cytochrome *c* oxidase (COX) is the terminal oxidase of the electron transfer system and couples the exergonic four-electron reduction of oxygen to pumping of protons across the membrane [57], which can be used to produce ATP. COX functions are inhibited by cyanide, azide and carbon monoxide [48]. COX deficiency has been associated with several diseases such as Alzheimer's and Parkinson's diseases. Because of the sequence differences in cytochrome *c* oxidase subunits in mammals and fungi, these proteins represent a potential target for the treatment of fungal infections [48, 58].

COX contains four redox-active metal centers (heme *a*, heme a_3, Cu_A, Cu_B) that participate in electron transfer. Heme a_3 and Cu_B are bridged in the resting fully oxidized form and constitute the binuclear reaction center. Electrons pass from cytochrome *c*, via Cu_A and heme *a*, to the binuclear reaction center, where O_2 is reduced to water. Cytochrome *c* oxidase is composed of 11 subunits: one copy each of COX1, COX2, COX3, COX4, COX5a or COX5b, COX6, COX7, COX9, COX12, COX13 and two copies of COX8. Extensive studies with the yeast subunit COX5 isoforms (*COX5a* and *COX5b*) have revealed that the genes for these proteins are switched on or off depending on the oxygen concentrations.

3.1.1.5. Complex V (ATP Synthase)

The ATP synthase is a complicated protein complex, known to be comprised of two separate multisubunit proteins: F_0 the trans-membrane part of the complex that allows protons to move down the gradient and F_1 the part of the complex that extends into the matrix and acts as the ATP synthesis enzyme. Complex V uses the proton gradient generated by the proton pumps to synthesize ATP. The exact number of protons required to synthesize a molecule of ATP are a matter of some controversy. Current estimates seem to indicate that about 4 H^+ must move down the gradient for each ATP produced. The spontaneous backflow of protons to the matrix through ATP synthase is coupled to the synthesis of ATP from ADP and P_i. Oligomycin is the specific inhibitor of complex V [59].

3.1.2. Alternative respiratory pathways

The second respiratory pathway present in *C. albicans* is the alternative oxidative pathway (AOX), which is generally conferred by a cyanide-insensitive alternative oxidase located on the matrix side of the inner mitochondrial membrane and encoded by two nuclear genes, *AOX1* and *AOX2*. The constitutive AOX1 or the inducible AOX2 bypass the CRC by transferring electrons directly from ubiquinol to oxygen without translocation of protons (Figure 8). The alternative respiratory pathway has been characterized in *C. albicans* and *C. parapsilosis* and was also detected in *C. utilis*. AOX2 is induced by inhibiting the CRC [60, 61]. In addition, some reports showed that reactive oxygen species, such as superoxide radical anion or hydrogen peroxide, induce the expression of AOX2 [62, 63]. These observations suggest that cyanide-resistant respiration is related to defense systems against oxidative stress. The function of the alternative respiratory pathway is inhibited by salicylhydroxamic acid (SHA) [49]. Under certain conditions, both the classical and

alternative pathways operate simultaneously [64], e.g. blocking the complex I activity by using rotenone. In terms of finding an antifungal target, the AOX was considered as a promising target because it is missing in humans.

Finally, the activation of PAR function only occurs when both the CRC and AOX pathways are blocked, allowing electron flux to be redirected upstream of complex III, in parallel to CRC [65]. KCN at higher concentrations is an inhibitor of both the PAR pathway and of complex IV of the CRC pathway [34].

3.2. Electron transport chain of S. cerevisiae

S. cerevisiae is a Crabtree-positive organism, which utilizes glucose repression of aerobic respiration to depend upon the fermentation as a source of energy. In *S. cerevisiae*, most elements of the classical respiratory chain are present, but complex I is absent (Figure 9). Instead, *S. cerevisiae* has three NADH dehydrogenases associated with the inner mitochondrial membrane capable of coupling the oxidation of NADH to the reduction of CoQ. One internal NADH dehydrogenase (NDI) faces the matrix space and utilizes mitochondrial NADH; the other two external NADH dehydrogenases (NDE1 and NDE2) face outward and can receive electrons from cytosolic NADH. These alternative dehydrogenases are insensitive to rotenone (the inhibitor of complex I) and lack Fe-S redox centers, while all are nuclear encoded with mitochondrial targeting sequences near the N-terminus. The internal rotenone-insensitive NADH-Q oxidoreductase (NDI1) of mitochondria was reported to be composed of a single subunit and to contain non-covalently bound FAD as a cofactor and no iron-sulfur clusters. Flavone was used to inhibit the enzymatic function of NDI1. The disruption of NDE1 led to reduced NADH dehydrogenase activity for cells cultivated in glucose, and the electron transfer from reduced NADH to O_2 electron transfer for isolated mitochondria. However, disruption of NDE2 had no effect [66, 67]. The major functional difference of the internal and external NADH-dehydrogenases from complex I is that electron transport to UQ is not coupled to proton translocation and therefore, there are only two protons pumping sites (complex III and IV) in *S. cerevisiae* electron transport chain. It's worth mentioning here that the respiratory chain structure of *C. glabrata* is similar to the respiratory chain structure of *S. cerevisiae* [68].

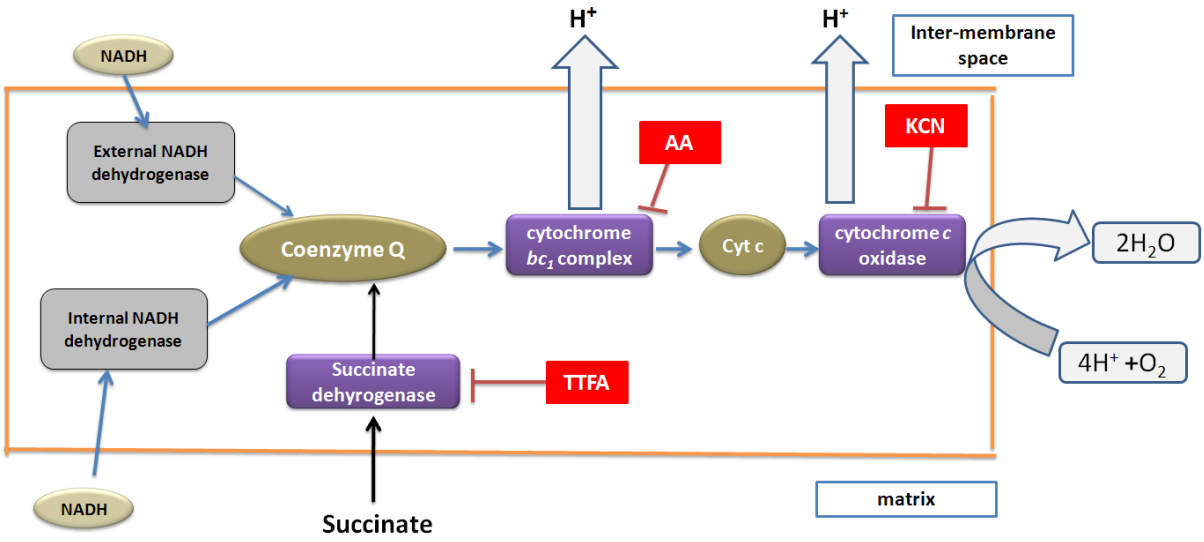

Figure 9: Schematic representation of the respiratory chain structure of *S. cerevisiae.* External and internal NADH dehydrogenases are alternatives for the complex I which is missing in *S. cerevisiae.*

4. Assays of respiratory chain activity

Any toxicant affecting the mitochondrial activity and cell energy budget will cause a defect in the cell function. The ability to evaluate the mitochondrial performance (function or dysfunctions) would lend insight into the diagnosis, treatment, prevention of some diseases and analysis of fungal pathogenic activity. The availability of a simple technique that could provide direct and precise information about the respiratory activity is needed. In the following section, the methods, biochemical and electrochemical, applied in this study are introduced in detail.

4.1. Oxygen uptake measurement

Measuring the oxygen uptake rates of the microorganisms during the cultivation process is one of the most informative tools for detecting the mitochondrial states. The polarographic method for monitoring oxygen uptake is one of the classical assays [69]. However, this method required a large volume of culture which leads to consume large amounts of the expensive inhibitors, moreover, the limited sample number per assay. The ability to easily measure the oxygen uptake in small volumes with low cell number is required. Particularly if efficacy of the respiratory chain of the target organisms is high, e.g. *C. albicans* which is able to consume the oxygen content in a very short time. Therefore, we developed a sensitive

method in order to measure the oxygen uptake rates of the tested organisms. To this end, the oxo-plate (96-well plate with integrated oxygen-sensor) was used [11]. The developed assay can be used for the direct measurement of oxygen uptake in microliter-scale volumes samples with fast or slow consumption rates (respiration rates).

Description of the oxygen-sensor microtiter plate

Oxo-plates (U-bottomed well-plates from PRESENS, Precision Sensing GmbH (Regensburg, Germany)) are sterile polystyrene micro-plates in the common 96-well format. The oxo-plate is supplied with a lid to keep the oxygen content constant during the cultivation process. An oxygen sensor is immobilized on the bottom of each well (Figure 10). The sensor consists of two different fluorescent dyes. One is the oxygen indicator, its phosphorescence intensity I_{ind} is dependent on the concentration of oxygen in the sample filled into the well. The other dye is the reference; its fluorescence intensity I_{ref} is independent of the oxygen concentration. This sensor can be read-out from the bottom with a commercially available fluorescence reader. The referenced signal I_R corresponds to the concentration of oxygen and is calculated by using equation 1:

$$\mathbf{I_R = I_{ind}/I_{ref}} \qquad \text{(equation 1)}$$

in each new experiment, calibration of the oxo- plate is required via the determination of zero point (0 % of O_2) by the chemical removal of dissolved oxygen using sodium sulfite and by saturation with oxygen (100 % of O_2).

Oxygen concentrations were calculated from equation 2.

$$pO_2 = 100\left(I_R^0/I_R - 1\right)/\left(I_R^0/I_R^{100} - 1\right) \qquad \text{(equation 2)}$$

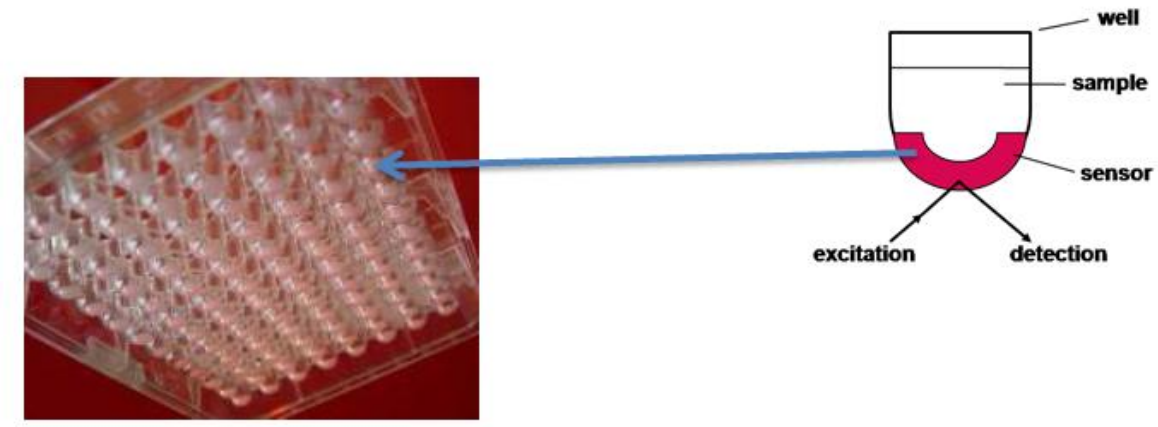

Figure 10: Dissolved oxygen measurement in round-bottomed microtiter plates (Oxo-plate) with an integrated oxygen sensor.

4.2. ROS Production by the respiratory chain

Reactive Oxygen Species (ROS) summarizes the reactive molecules and free radicals derived from molecular oxygen. ROS are known to be released by phagocytic cells as a part of their role in host defense. Recent work has demonstrated that ROS have a role in cell signaling, including apoptosis gene expression and the activation of cell signaling cascades [70]. ROS are generated in mitochondria as a normal byproduct of aerobic metabolism; 0.2–2% of O_2 consumption is estimated to be lost as superoxide under normal conditions [71]. ROS generation by the electron transport chain (ETC) is critically dependent upon ETC redox state, and the disturbance of the normal redox state of mitochondria leads to a production of ROS that can cause damage in components of the living system. Mitochondrial dysfunction may lead to an increase in the production of radicals and consequent to oxidative stress. Under normal conditions, the damaging effects of ROS are neutralized by enzymes such as glutathione (GSH) peroxidases and superoxide dismutase (SOD), thereby preventing further cellular damage. But under certain conditions (e.g. blocking the respiratory functions) that will lead to stress, the amount of formed ROS will exceed the handling capacity of the cell's antioxidant mechanisms, resulting in oxidative stress.

Concerning the nature of the superoxide generators in the respiratory chain, mitochondrial complexes I (NADH:ubiquinone oxidoreductase) and complex III (ubiquinol:cytochrome *c* oxidoreductase) are generally regarded as the main sources of ROS (Fig. 11) [72]. In complex I, superoxide is primarily produced at the bound flavine facing the matrix side. In the

cytochrome *bc*1 complex the superoxide is formed at the ubiquinol oxidation site (Q_o site, center P) facing the intermembrane space [72-74].

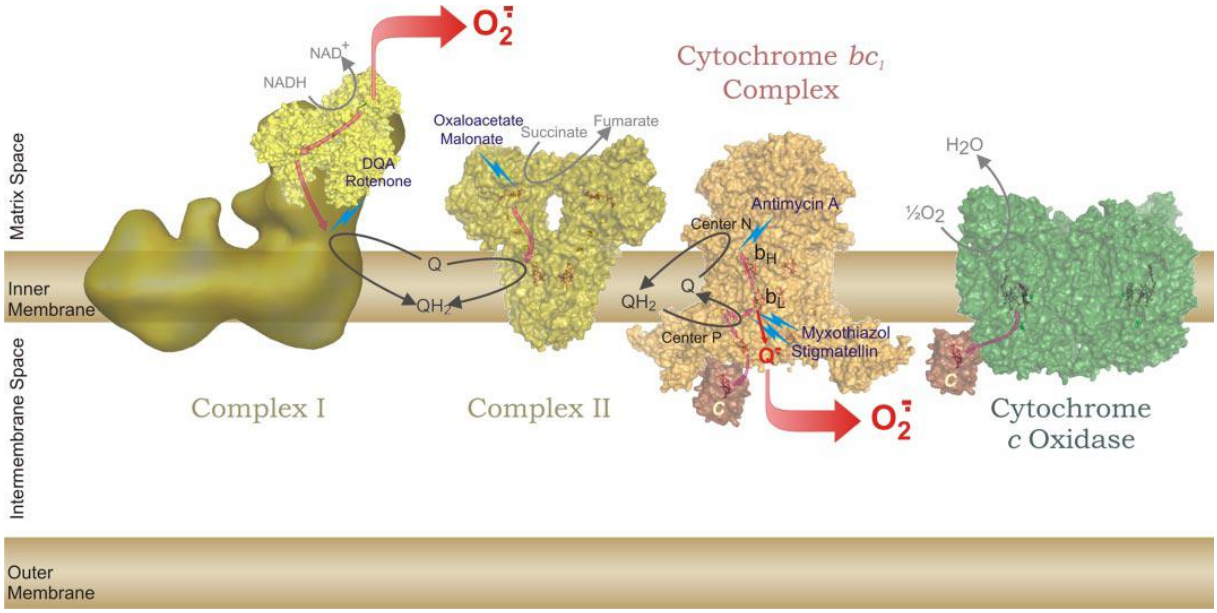

Figure 11: Superoxide generation by the respiratory chain. Complex I and complex III are the major sources of ROS. In complex I, superoxide is primarily produced at the bound flavine facing the matrix side, whereas in the cytochrome *bc*1 complex superoxide is formed at the ubiquinol oxidation site (Q_o site, center P) facing the intermembrane space [72].

There are a number of different compounds that can be used to assess the mitochondrial ROS production. However, to measure the ROS in the living cells the used reagents must cross the intact cell membranes to be able to react with the superoxides. Therefore, we used H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate, acetyl ester), SE as ROS indicator [75].

H_2DCFDA is widely used to measure oxidative stress in cells. H_2DCFDA is a membrane permeable molecule. Once inside the cell, cellular esterases act on the molecule to form the non-fluorescent moiety H_2DCFDA. Oxidation of H_2DCFDA by ROS converts the molecule to 2',7' dichlorodihydrofluorescein (DCF), which is highly fluorescent (Figure 12). Upon stimulation, the resultant production of ROS causes an increase in fluorescence signal over time.

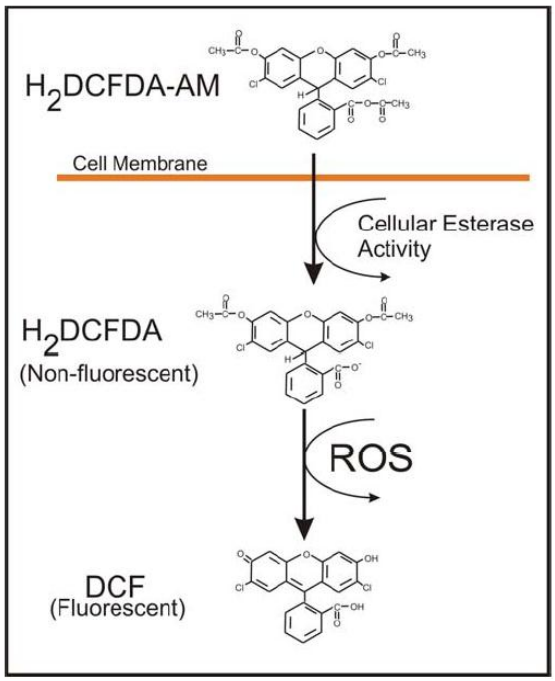

Figure 12: Formation of fluorescent compound DCF by ROS [76]

4.3. The use of tetrazolium salts to detect the mitochondrial activity

Colorimetric assays of cellular viability are important tools in the study of microorganism's activity. The most common colorimetric assays are based on the use of tetrazolium salts [77, 78], and have evolved since the description of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay in the early 1980s [78]. Basically, the oxidized form of the tetrazolium salt is reduced by electron transfer catalyzed by mitochondrial dehydrogenases in metabolically active cells which then leads to the production of formazans (highly coloured end products) [79]. The amount of formazan is directly proportional to the activity of the cells, present during the tetrazolium exposure.

In this work, the tetrazolium salt WST-1 (4-[3-(4-Jodophenyl)-2-(4-nitrophenyl)-2H-5-Tetrazolio]-1.3-Benzoldisulfonate) was used. The reduction of WST-1 by the viable yeast cells results in a water-soluble yellow formazan, which can be easily quantified at the wavelength of 450 nm [80].

4.4. DCIP assay

The activity of NADH dehydrogenases, in particular of complex I of the respiratory chain was determined via the detection of 2,6-dichlorophenolindophenol (DCIP). DCIP is one of the compounds allowing both spectrophotometric [81, 82] and electrochemical [83] detection

of cellular reactions. It is known as photometric pH and redox indicator [84]. The reduced form of DCIP is colorless, whereas the oxidized form has a dark blue color in solutions with pH > 6 and is pink in solutions with lower pH. The reduced form of DCIP contains two oxidizable groups, namely the 2,6-dichloro-4-quinone imino group and the phenolic ring. The oxidized form of DCIP easily penetrates cell membranes and reacts with the intracellular NAD(P)H pool. This reaction is catalyzed by NAD(P)H dehydrogenases and DCIP is used as an indicator of NADH dehydrogenase activities, in particular the activity of complex I (Figure 13). However, DCIP was also used in a toxicity assay based on the complex I – negative yeast S. *cerevisiae*, when glucose was used as a carbon source [81, 85].

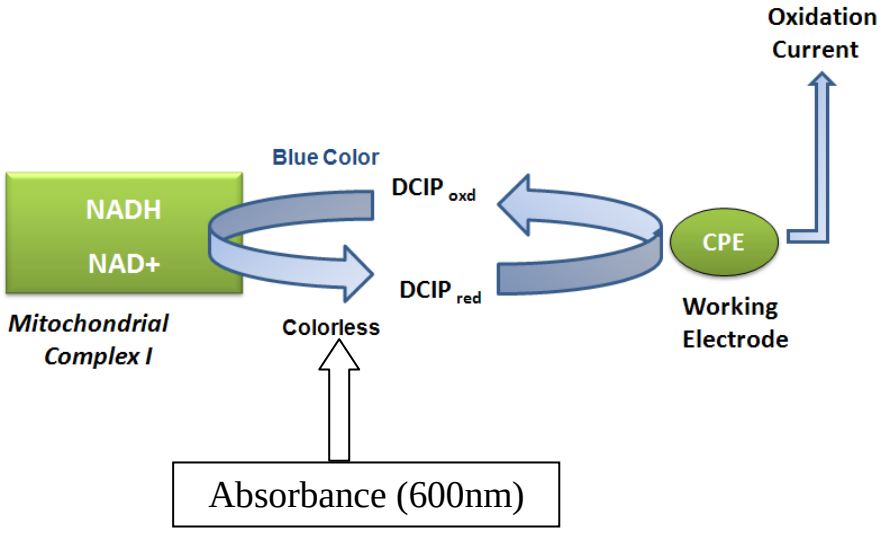

Figure 13: Schematic representation for the interaction of DCIP with *C. albicans* and suitable detection principles. The carbon paste electrode (CPE) was employed as a working electrode in the electrochemical systems.

4.5. Bio-electrochemical approach for evaluating the mitochondrial performance

Electron transfer reactions play a central role in all biological systems because they are essential to the processes by which biological cells capture and use energy. These electrons can be transferred also to electrodes leading to generation of an electrical current, which is named bioelectrochemical system.

In the bioelectrochemical system (BES), microorganisms catalyze electrochemical reactions through interactions with electrodes. In microbial fuel cells (MFCs) microbial activity is

exploited to generate electricity from various organic substrates (such as glucose or other carbon sources) [86].

Basically, microorganisms rely on the oxidation of the organic matters for energy production and thus have to transfer electrons to suitable electron acceptors. Examples of natural electron acceptors are oxygen, pyruvate, nitrate, and even metal oxides depending on the availability of oxygen and the capability of anaerobic respiration.

In BES, electrons are transferred from microorganisms to solid electrodes generating the electrical current. The density of the electrical current coming from the microorganisms is dependent on the concentrations of the electron transfer agents and on the efficiency of the electron transfer. Whereas the first are influenced by properties of the cell culture, such as cell numbers or the activity of the electron transport chain, the latter depends on the electrochemical properties of the electron transferring compound and on its steric accessibility, i.e., the physical contact between the respective redox center and the electrode. As the oxidation of carbon sources is the major source of electrons, the most important electron transfer system in organisms is the electron transport chain linked to respiration or fermentation pathways. In prokaryotic systems the respective proteins and electron shuttles are located in the plasma membrane, whereas in eukaryotic cells, they are present in the mitochondria. Thus, the bacterial electron transport chain is more easily accessible than that of eukaryotes, and most reports of microbial fuel cells deal with the development of bacterial bioanodes [87]. However, the use of yeasts to generate electrical current has also been suggested [88] as they can grow on cheap substrates and do not require anaerobic conditions.

4.5.1. Extracellular electron transfer: Electron transfer mechanisms

In our study, we did not use the bio-electrochemical systems just as tools to detect the mitochondrial performance, but we were also very interested in understanding the electron transferring mechanisms from the yeast mitochondria to the electrode system. Mechanisms for transferring of electrons from the microbial intracellular part to the surface of the working electrode have been studied extensively in the bacterial system, because the use of bacteria is more common in the microbial fuel cell technology. These studies have shown that electron transfer to extracellular solid materials, such as anodes and metal oxides, occurs either directly or indirectly via electron mediators (natural mediators or artificial mediators) [89].

4.5.1.1. Direct electron transfer (DET): Mediatorless transfer system

Organisms such as *Shewanella putrefaciens* [90], *Geobacter sulfurreducens* [91, 92], *Geobacter metallicreducens* and *Rhodoferax ferrireducens* [93, 94] have been shown to generate electricity in mediatorless MFC systems. There are two ways for the microbial cells to directly communicate with the solid electrode (the terminal electron acceptor) in order to generate the electrical current.

(A) The presence of the electro-active compounds (*cytochromes* for example) at the outer-cell membrane. These cytochromes are responsible for the communication of the microorganisms with the electrode [95, 96].

(B) Under specific conditions (e.g. biofilm formation), some bacteria (*Geobacter* and *Shewanella)* are able to produce conductive nano-wires (*Pili*) as a main connector between the microorganisms and the electrode surface [97-99].

4.5.1.2. Indirect electron transfer: Mediated electron transfer (MET)

When the direct electron transfer is not possible, the mediated approach can be a good alternative. In this case, the mediated electron transfer can be carried out either by:

(A) Self-mediated system;

A number of microbial species are capable of self-mediating electron transfer by producing and excreting small electron shuttles (*endogenous* chemical mediators), which are oxidized at the electrode surface and reduced inside the cell. For example, *Pseudomonas* species were shown to produce pyocyanin and phenazines [100-102], and *Shewanella* species produced flavins which serves the same purpose [103].

(B) Artificial-mediated system;

Artificial mediators can be used to improve the electron transfer across the cell membrane [103, 104]. They may be organic dyes (e.g. thionin, neutral red, 2,6-dichlorophenolindophenol (DCIP), methyl viologen, methylene blue), inorganic complexes (e.g. ferricyanide) or organometallics (osmium polymers) [105, 106].

However, there are some important requirements that such a mediator should satisfy in order to provide an efficient electron transport from the microbial intracellular part to the electrode surface:

(a) The oxidized state of the mediator should easily penetrate the cell membrane to reach the reductive species inside the microorganism.

(b) The reduced state of the mediator should easily escape from the cell (after reduction) through the cell membrane.

(c) The mediator should be chemically stable in the electrolyte solution (in the electrochemical cell) and be well soluble [101].

In this study, two bio-electrochemical approaches were established (chapters 3 and 4) for determining yeast's mitochondrial performance. These approaches were based on the mediated electron transfer system, either by using the self-mediated (secretion of tryptophol) or the DCIP-mediated systems.

Figure 14 summarizes the different bio-electrochemical approaches which have been developed and investigated in this study.

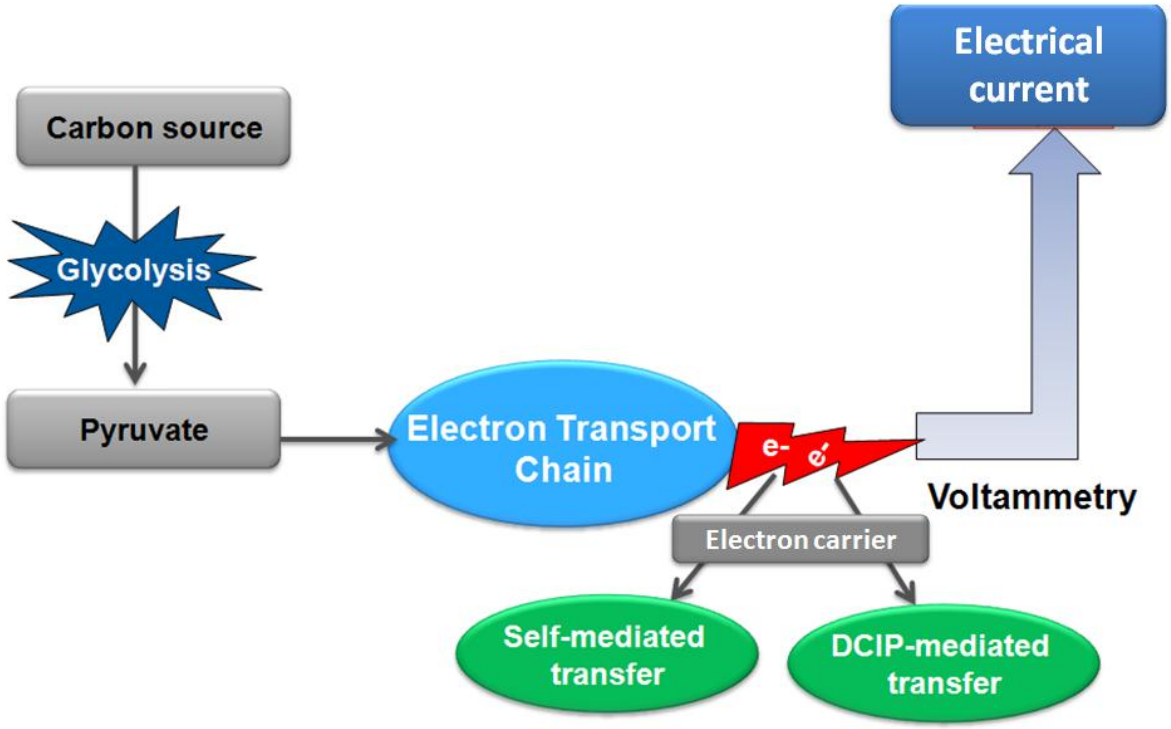

Figure 14: Yeast-electrochemical approaches which have been investigated and discussed in this study (Chapters 3 and 4).

4.5.2. Why do we need bio-electrochemistry?

Analysis of the mitochondrial complex I and IV activities, without the isolation of mitochondria and enzyme purification, is difficult using classical biochemical assays. Therefore, alternative techniques are required for completing the main objective of the study.

Moreover, the bioelectrochemical methods were used for the detection of viability of *C. albicans* and discrimination between electron transport chain activity of *C. albicans* and other fungi (*C. glabrata* and *S. cerevisiae*).

4.5.3. Electrochemical devices: Voltammetric technique

The electron transfer by yeast cells was demonstrated using the cyclic voltammetry (CV) and linear sweep voltammetry (LSV).

Voltammetry is an analytical technique based on the measure of the current flowing through an electrode immersed in a solution containing electro-active compounds (analyte). Variable electrode potentials are applied and the resultant current is measured through a three-electrode system (reference, working and auxiliary electrodes). The resulting voltammetric current reflects the concentration of the electro-active compound.

Cyclic Voltammetry (CV)

CV is the most widely used technique for acquiring qualitative information about electrochemical reactions. The power of cyclic voltammetry results from its ability to rapidly provide considerable information on the thermodynamics of redox processes and the kinetics of electron transfer reactions and on coupled chemical reactions or adsorption processes. In particular, it offers a rapid location (the redox potential, *Ep*) of the electroactive species. Cyclic voltammetry depends of scanning linearly the potential of a stationary working electrode (in an unstirred solution), using a triangular potential wave-form. During the potential sweep, the potentiostat measures the current resulting from the applied potential (*Ip*). The resulting current-potential plot is termed a cyclic voltammogram, as shown in Figure 15 [107].

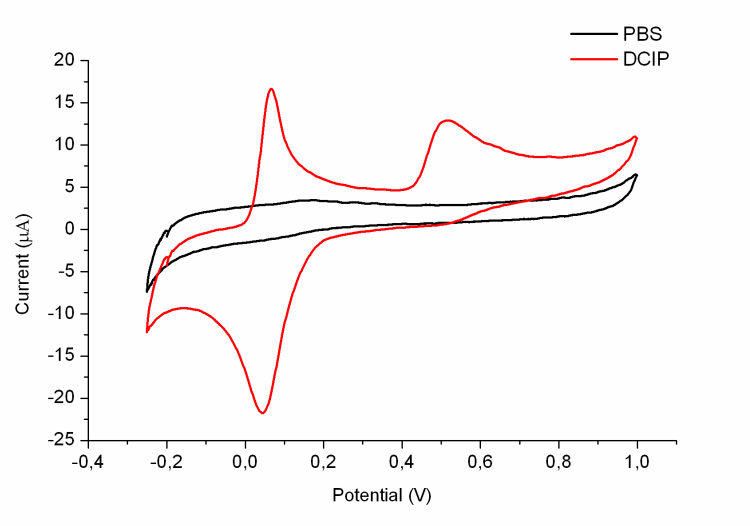

Figure 15: Typical cyclic voltammogram of DCIP in PBS buffer.

The voltammetric cell

Most electrochemical cells require at least two electrodes, since the potential of a given electrode can only be measured relative to another electrode, the potential of which must be constant (a reference electrode). However, in the voltammetric experiment, an external potential is applied to the cell, and the current response is measured. Precise control of the external applied potential is required, but this is generally not possible with the two electrode system, resulting from the potential drop across the cell due to the solution resistance and the polarization of the counter electrode that is required to complete the current measuring circuit. Better potential control is achieved using a potentiostat and the three electrode system (Figure 16), in which the potential of one electrode (the working electrode) is controlled relative to the reference electrode, and the current passes between the working electrode and the third electrode (the auxiliary electrode) [108].

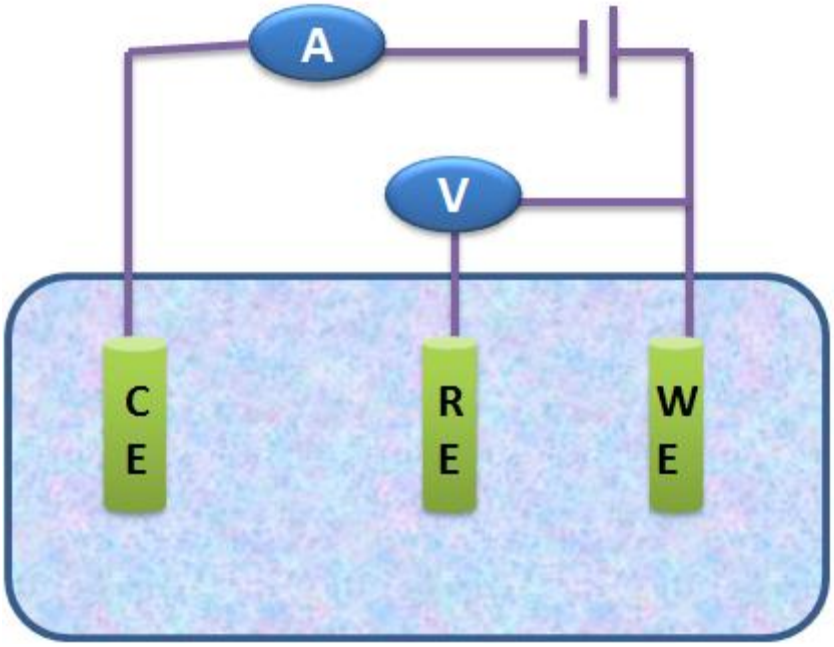

Figure 16: Schematic diagram of the three electrode setup, whereas (CE) is a counter electrode, (RE) a reference electrode and (WE) a working electrode

The working electrode: Carbon Paste Electrodes

Carbon paste electrodes which use graphite powder mixed with various water-immiscible non-conducting organic binders (paraffin oil used as pasting liquids), offer an easily renewable and modified surface, low cost, and very low background current contributions [109]. The paste composition strongly affects the electrode reactivity, with the increase in pasting liquid content decreasing the electron transfer rates, as well as the background current contributions [110]. In the absence of pasting liquid, the dry graphite electrode yields very rapid electron transfer rates.

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Design of a new strategy for respiratory chain inhibitors: understanding mechanisms of action

Abstract

The severity of infections caused by *Candida albicans*, the most common opportunistic human fungal pathogen, needs rapid and effective antifungal treatments. As the respiratory chain is involved in the regulation of many of the fungal virulence factors it offers attractive potential antifungal target. Due to the complex structure of the electron transport chain and the metabolic flexibility of *C. albicans*, the biological activity of complex I and IV inhibitors could not be detected by the growth inhibition test or by measuring oxygen consumption rates. Therefore, the current study introduced a complementary strategy, consisting of several biochemical assays, to elucidate the function of respiratory chain components and to identify the site of action of chemical compounds acting on the respiratory chain. We used the most common respiratory chain inhibitors acting at different binding sites, namely rotenone, TTFA, antimycin and KCN; as complex I to IV inhibitors respectively and SHA as AOX inhibitor. Additionally, two secondary metabolites, myxothiazol and pyrrolnitrin, were included which act on complex III and on the early steps of the electron transport chain.

The influences of these compounds on *C. albicans* were analyzed by several biochemical assays.

1. Monitoring of growth rates on different types of carbon sources allows detecting whether an essential complex or function is inhibited.
2. Determination of oxygen consumption rates shows the overall activity of terminal oxidases.
3. Determination of reactive oxygen species production predicts whether the complex I or complex III is the targeted.
4. The metabolites quantification of ethanol and glycerol is used as indicator of the activity of the respiratory vs the fermentation metabolic pathways.
5. The viability of the yeasts was detected via WST-test, which mainly indicates the metabolic activity of the yeast cells.

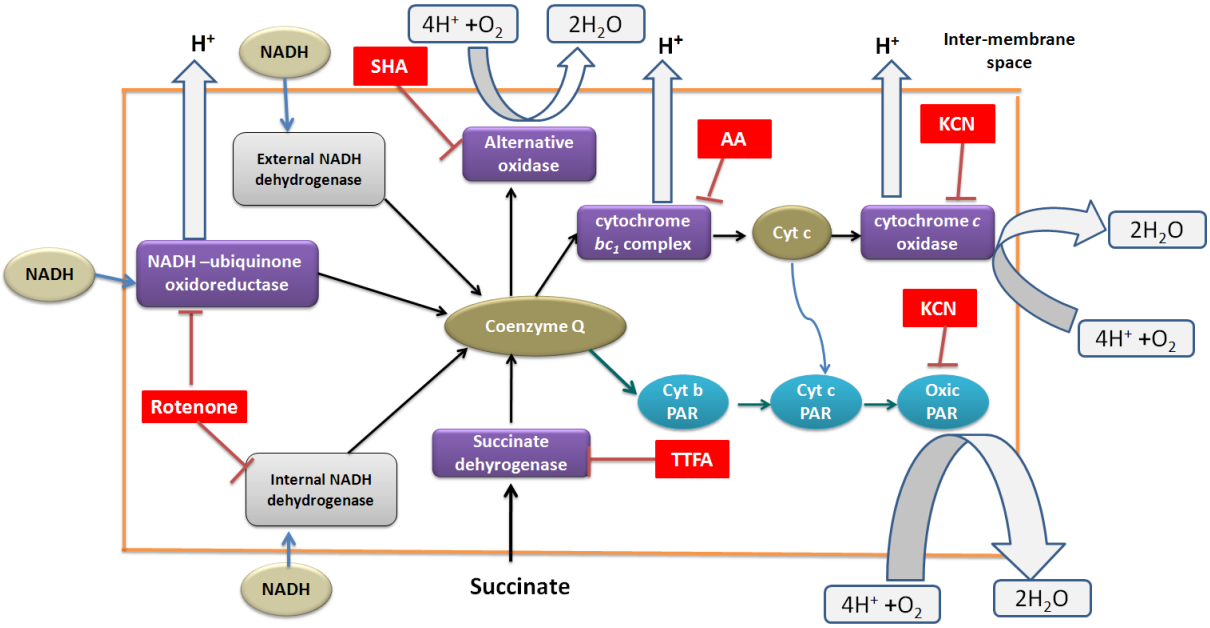
1. Introduction

Different kinds of mycoses, especially invasive, have become an important public health problem as their occurrence has increased dramatically in the last decades associated with AIDS, transplant recipients and other immunosuppressed individuals [1]. Immunocompromised patients are mainly infected by *Candida*, *Aspergillus*, *Cryptococcus* spp. and other opportunistic fungi, while, *Candida albicans* the most frequently found fungal pathogens. The handling of the fungal infection is limited by drug safety, resistance development and limited effectiveness [1]. Therefore, continuous efforts are required to discover new compounds with appropriate biological activities [2, 3]. The electron transport chain is one of the most important biochemical cascade reactions in the energy supply of the aerobic organisms. The enzymes, which mediate electron transfer and ATP synthesis in a sequence of redox reactions of oxidative phosphorylation, are exclusively located in mitochondria (in eukaryotic organisms) or bacterial membranes [4, 5]. The best-known function of the mitochondrial respiratory chain is its ability to generate the vast majority (more than 90%) of cellular energy in the form of ATP, which is essential to drive and maintain physiological activities [6].

The classical respiratory chain structure in eukaryotes comprises three large protein complexes: NADH-ubiquinone oxidoreductase (complex I), ubiquinol cytochrome *c* oxidoreductase (complex III or cytochrome bc_1 complex) and cytochrome *c* oxidase (complex IV). In each complex electron transport is coupled to proton translocation, with the resultant proton motive force (pmf) being used for ATP synthesis. Each complex can specifically be inhibited, e.g. complex I by rotenone, complex III by antimycin and myxothiazol and complex IV by cyanide. Electron transfer from succinate to ubiquinone without pumping of protons is achieved by succinate-dehydrogenase complex II, of which thenoyltrifluoroacetone TTFA is a specific inhibitor [7].

However, *C. albicans*, the most common human fungal pathogen, has a more complicated respiratory chain structure due to the presence of three respiratory pathways: the classical respiratory chain (CRC), the alternative respiratory chain and the parallel electron transport chain (PAR) [8, 9], (Scheme 1). The CRC comprises the four enzymatic complexes as described before [10]. The alternative respiratory pathway is generally conferred by a cyanide-insensitive alternative oxidase located on the matrix side of the inner mitochondrial membrane and encoded by two nuclear genes, *AOX1* and *AOX2*. *AOX1* is constitutively expressed, whereas expression of *AOX2* is induced, for example by the presence of the CRC

inhibitors. These oxidases catalyze the direct oxidation of ubiquinol by oxygen without proton translocation and, thus, enable respiration even in the presence of CRC inhibitors. The catalytic function of the alternative oxidative pathway is inhibited by salicylhydroxamic acid (SHA) [11]. PAR is only activated when both the CRC and AOX pathways are totally blocked, allowing electron flux to be redirected upstream of complex III, parallel to the CRC [9, 12].

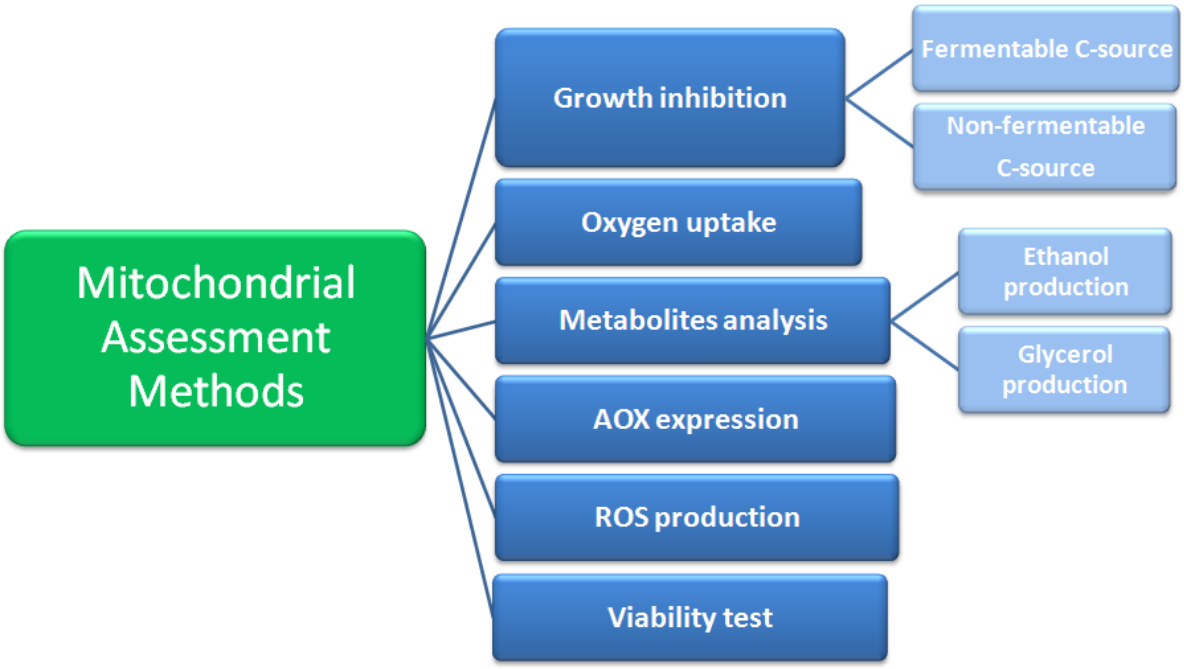


Scheme 1: Respiratory chain structure of *C. albicans.* NADH is oxidized by any of the NADH-dehydrogenases leading to reduced coenzyme Q. This is also produced during the oxidation of succinate by succinate-dehydrogenase. Reduced coenzyme Q is re-oxidized either by oxygen through the alternative oxidase, or by cytochrome c in the cytochrome bc_1 complex. Cytochrome *c* is finally oxidized by cytochrome *c* oxidase utilizing oxygen. These latter reactions can also be catalyzed by components of the parallel pathway PAR.

In other fungi, such as the pathogenic yeast *Candida glabrata* and the non-pathogenic yeast *Saccharomyces cerevisiae*, the mitochondrial complex I is absent and the so-called external and internal NADH dehydrogenases catalyze the oxidation of NADH without generation of a trans-membrane proton gradient [10, 11, 13, 14]. These alternative dehydrogenases are usually rotenone-insensitive.

The electron transport chain enzymes are considered to be potential drug targets due to the involvement of these enzymes in many biological process besides energy gain (e.g. hyphal induction, biofilm formation, oxidative stress and other fungal virulence factors) [15]. Identification of affected proteins and of binding sites of new respiratory chain inhibitors is necessary in drug discovery. We have reported that the effect of respiratory chain inhibitors on yeast cells can be easily detected by measuring oxygen uptake as growth is not affected in rich media [16]. However, in *C. albicans* the influence of the complex I and IV inhibitors could not be detected by this way. Therefore, additional assays are required and this study aimed at a complementary strategy, which consists of several biochemical assays delivering data on the activity, and functionality of different respiratory pathways and complexes. Consequently, this assay combination serves as a platform to identify the mechanisms of toxicity for the electron transport chain inhibitors.

In order to achieve the strategic objective, the growth rate, oxygen consumption, AOX expression, cell viability, ROS induction and ethanol/glycerol production of *C. albicans* and *S. cerevisiae* were studied after incubation with the classical respiratory chain inhibitors: rotenone, TTFA, antimycin A, and KCN. SHA was also included as the alternative oxidase inhibitor. Moreover, the natural products myxothiazol and pyrrolnitrin were used. Myxothiazol was extracted from the myxobacterium, *Myxococcus fulvus*, and used to inhibit the mitochondrial complex III [17, 18]. Pyrrolnitrin was isolated from *Pseudomonas pyrrocinia* and *P. aureofaciens* and its activity supposedly lies in the ability to block electron transfer of *S. cerevisiae* between succinate or NADH and coenzyme Q [19, 20]. Further studies showed that pyrrolnitrin not only acts as ETC-inhibitor but also interferes with the osmotic signal transduction pathways in *Neurospora crassa* [21].



Scheme 2: The working-steps of the current strategy were planned as the following: Starting the study by investigating the effects of respiratory chain inhibitors on the growth (growth inhibition test) and in this step the type of carbon source, C-sources, (fermentable or non-fermentable) has to be considered. Then, the mitochondrial efficacy was evaluated by measuring the oxygen uptake rate; afterwards, effects of ETC-inhibitors on the activation of the alternative pathway (AOX) were analyzed. Consequently, the ability of the treated cells to switch from cellular respiration to alcoholic fermentation was examined on different carbon sources (fermentable and non-fermentable). Furthermore, stimulation of the cellular ROS production by treated cells was determined, and the viability of cells was recorded.

2. Material and methods

2.1. Chemicals and reagents

Myxothiazol was provided B. Kunze (KOM, HZI, Braunschweig, Germany) and pyrrolnitrin by K. Gerth, H. Steinmetz and R. Jansen (MWIS, HZI, Braunschweig, Germany).

Rotenone, antimycin A, 2- thenoyltrifluroacetone (TTFA) and KCN were obtained from Sigma, Fluka, and Roth, respectively. Salicylhydroxamic acid (SHA) was from Sigma-Aldrich.

Alcoholdehydrogenase (ADH), glyceroldehydrogenase (GDH) and NAD^+ for the enzymatic assays of ethanol and glycerol were obtained from Fluka, Sigma and Biomol, respectively. Cultivation media YPD, YNB (with amino acids) and YNB (without amino acids) were obtained from Sigma and RPMI (1640) with L-Glutamine was from Lonza. WST-1 (4-[3-(4-Jodophenyl)-2-(4-nitrophenyl)-2H-5-Tetrazolio]-1.3-Benzoldisulfonate) for the viability assay was purchased from Roche.

2.2. Strains and growth conditions

The following microbial strains were used: *Candida albicans* CAF2-1 [22, 23] and *Saccharomyces cerevisiae* BY4741. Yeast cells were cultivated overnight in 250 ml flasks in 50 ml YPD medium (yeast extract (10 g/l), peptone (20 g/l), and glucose (20 g/l)) at 30° C. A pre-culture was prepared by diluting the overnight culture to an optical density (OD_{620}) of 0.2 in 25 ml YNB (2% glucose). Yeast Nitrogen base with amino acids was used for cultivation of *S. cerevisiae*. YNB without amino acids was used for cultivation of *C. albicans*. The yeast cells were allowed to grow for 3 hours so that they reached the exponential growth phase. The OD_{620} was determined in 180 µl sample volume with the microtiter plate reader µQuant (BioTek Instruments GmbH, Bad Friedrichshall, Germany). Subsequently, the working culture was prepared by diluting the pre-culture to the desired OD.

2.3. Measuring of oxygen consumption

The oxygen uptake rate was monitored during the cultivation of the chosen yeasts in YPD according to our previously described procedure [24]. Oxygen was determined with round-bottomed oxo-Plates (PreSens, Regensburg, Germany) following the procedure given by the manufacturer. A working culture was prepared from the pre-culture by dilution with YPD to OD_{620} of 0.05 for *C. albicans* and 0.1 for *S. cerevisiae*.

Each well of the oxoplate (96 wells) was filled with 60 µl YPD, and 30 µl of the stock solution of a compound was added to the wells in the first column (1: 3 dilution of the stock solution). Then, nine times 30 µl solutions were transferred from one column to the following columns, resulting in a serial 1: 3 dilution. From the 10^{th} column, 30 µl was discarded to obtain a final volume of 60 µl. For each compound of the ETC-inhibitors, three rows were used, so that two compounds were combined on one microtitre plate. The remaining two rows were used for the solvent as a control, of which 30 µl were added to the first column and also serially diluted. Column 11 contained only medium (untreated condition) and column 12 was used for calibration of the oxo-plates, so that neither medium nor compounds were filled in. Finally, 120 µl of the yeast working culture were added to each of the wells in the 11 columns leading to another 1: 3 dilution step of the compounds.

Then oxygen was determined fluorimetrically at different cultivation times. The fluorescence of the indicator and the reference dye was determined through the bottom of the plates at excitation wavelengths λex of 530 nm and emission wavelengths λem of 620 nm for the indicator dye and λex 530 nm and λem 590 nm for the reference dye. The oxo-plate calibration was performed using water saturated with air (100%) and using an aqueous Na_2SO_3 solution (10 g/l) (0%) according to the supplier's protocol.

2.4. Measurement of Reactive Oxygen Species (ROS)

The amount of reactive oxygen species (ROS) produced in the yeast cells was measured by a fluorimetric assay using H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate, acetyl ester), SE (Invitrogen) as a ROS indicator as previously described [25]. A 96 well plate with different concentration of the ETC-inhibitors was prepared according to the protocol given in section 2.3 for the oxoplate besides the use of MQ water instead of YPD. The working culture was prepared by diluting the pre-culture to an OD_{620} of 0.2 in fresh YPD. After cultivation for another 3 hours, the OD_{620} was measured and cells were harvested by centrifugation for 5 min at 5000 rpm at room temperature. They were washed carefully three times with MQ water. Washed cells were resuspended in sterile MQ water to an OD_{620} of 0.5. The H_2DCFDA was added from a stock solution of 2 mg/ml in DMSO and diluted to a final concentration of 40 µg/ml. After 30 min of incubation at 30 °C, the stained cells were again collected by centrifugation and re-suspended in sterile MQ water. 120 µl of the stained cells were transferred into each well of the 96 well-plate containing 60 µL of ETC-inhibitors. The fluorescence intensity was quantified after 2 h, using a fluorescence microtiter plate reader

(Synergy 4, BioTek Instruments GmbH) at the excitation wavelength λex 485 nm and the emission wavelength λem 535 nm.

The percentage of ROS stimulated by mitochondrial inhibitors was calculated as follows:

ROS induction (%) = ($F_{Treated}$ / $F_{Control}$)*100 (equation 1)

Whereas $F_{treated}$ is the fluorescence obtained from the cells treated with ETC-inhibitors, and $F_{control}$ is fluorescence obtained from the cells treated with the solvent.

2.5. Selection of a single concentration of each inhibitor

A single concentration of each inhibitor was selected on the basis of results of the determination of the oxygen uptake and ROS production. The following experiments (growth inhibition test, AOX expression, ethanol/glycerol production and the viability test) were performed using one single concentration.

Table 1: Concentration of different respiratory chain inhibitors

Compound/ (Abbreviation)	Concentration (µg/ml)	Inhibition of mitochondrial function	
Rotenone (Rot)	41	Complex I	NADH-ubiquinone oxidoreductase
Thenoyltrifluroacetone (TTFA)	5	Complex II	succinate-dehydrogenase
Antimycin A (AA)	1.5	Complex III	Cytochrome *bc1*
Salicylhydroxamic acid (SHA)	13	AOX	Alternative Oxidase
KCN	5	Complex IV	cytochrome c oxidase
Myxothiazol (Myxo)	5	Complex III	Cytochrome *bc1*
Pyrrolnitrin (Pyrrol)	13	In between (CI-CII)	Succinate or (NADH) and coenzyme Q

2.6. Gene expression analysis of AOX1 and AOX2 in C. albicans

(This experiment has been performed by Anna Buschart, BiSA, HZI)

20 ml of working cultures of *C. albicans* were prepared in YPD and supplemented with the ETC-inhibitors using the final concentrations given in Table 1. After an incubation time of 20 min at 30 °C, the cells were harvested by centrifugation and the cell pellets were shock frozen in liquid nitrogen. Still-frozen cells were suspended in 0.6 ml RLT buffer (Qiagen) and mechanically disrupted using glass beads (425-600 µm, Sigma). RNA was isolated on RNeasy mini columns with added DNAse (Qiagen) as recommended by the manufacturer. The quality and integrity of total RNA from a subset of the samples was controlled with the Agilent Technologies 2100 Bioanalyzer (Agilent Technologies).

2 µg of total RNA were reverse-transcribed by superscript II reverse transcriptase, with random and oligo-dT12-18 primers, according to the manufacturer's recommendations (Invitrogen). Quantitative real-time PCR was carried out on a 384-well LightCycler® 480 system by using the LightCycler® 480 SYBR Green I Master (Roche). Gene sequences were obtained from the *C. albicans* Genome Database [26] and gene-specific oligonucleotides (Table 2) were designed by Roche's ProbeLibrary Assay Design Center and synthesized by Eurofins MWG Operon. Specificity was controlled against the *C. albicans* genome sequence using BLAST. PCR cycling parameters were: 95 °C, 60 °C and 72 °C for 10 s each for 45 cycles, with a subsequent melting-curve analysis. Real-time analysis data (crossing-points) were normalized with respect to the actin gene *ACT1* and used to calculate relative gene expression levels. The averages and standard deviations of the gene expression levels relative to solvent controls in three independent experiments were calculated, and the significance of the changes in gene expression was tested by Student's t-test of normalized data ($p < 0.05$).

Table 2: Primers used for real-time PCR analysis of gene expression.

Target gene	**Oligonucleotide**	
	Name	**Sequence**
CaACT1	*CaACT1*left	aaccaccggtattgttttgg
	*CaACT1*right	gcgtaaattggaacaacgtg
CaAOX1	*CaAOX1*left	cccgaatcaagcttcaaaga
	*CaAOX1*right	attagcaaacgtatggttaatttctct
CaAOX2	*CaAOX2*left	cctagatattgtcatagattcgttgg
	*CaAOX2*right	tttcccaggaacagcaagtt

2.7. *Determination of ethanol and glycerol*

2.7.1 Sample preparation

The time course of ethanol and glycerol production was followed during cultivation of *C. albicans*. 25 ml of YNB (+ 2% glucose) was placed in a shake flask, and then an appropriate volume of each inhibitor was added to each flask using the final concentrations which are shown in Table 1. Cultivation media supplemented with the respective volume of the solvent were used as control.

An appropriate volume of the pre-culture (which makes the working culture OD_{620} equal to 0.2) was centrifuged for 2 min at 5000 rpm. The supernatant was discarded to remove metabolites from the pre-culture and the cells were suspended in the media of the working culture flaks. Cultures were grown at 30 °C while orbitally shaken (160 rpm) for at least 5 h.

Samples of 200 µl were taken each hour, the OD_{620} was determined; the sample was transferred to an Eppendorf tube (1 ml) and centrifuged at 14000 rpm (Eppendorf centrifuge 5804R) for 10 min at 4°C. The supernatant was collected and frozen at - 20 °C until use.

Both ethanol and glycerol concentrations were determined by enzymatic assays based on alcohol (ADH) dehydrogenase and glycerol dehydrogenase (GDH), as illustrated in equations 1 and 2. Quantification in supernatants was based on the photometric determination of NADH at 340 nm, and the assays were performed in volume reduced 96 well transparent microtiter plates (Corning). Standard calibration curves were designed. For glycerol, the

concentration range from 0 to 10 mM was covered and for ethanol from 0 to 100 mM. To avoid evaporation of liquids during preparation of microtiter plates, the solutions were placed on ice.

2.7.2. Ethanol determination

All solutions of the enzyme mixture were prepared in 100 mM sodium pyrophosphate buffer, for which 4.46 g of sodium pyrophosphate were solved in 100 ml MQ water. The pH was adjusted with phosphoric acid. The enzyme mix comprised alcohol dehydrogenase (ADH; 288 U/mg; stock solution: 3 U/µl NaPP, pH 7.5) and NAD^+ (stock solution: 11 mM in NaPP, pH 8.5). For the preparation of the enzyme mix, 33 µl of ADH were added to 5 ml NAD^+ solution. Ethanol standards were prepared in YNB. 5 µl of the sample or the standard solution were placed in each well, and the reaction was started by adding 45 µl of the enzyme mix. After incubation at room temperature for 30 min, the absorbance at 340 nm was determined using the microtiter plate reader (µQuant, Biotek). Four aliquots of each sample were analyzed.

2.7.3. Glycerol determination

An enzyme mix was prepared, which contained the enzyme glycerol dehydrogenase (GDH from *Cellulomonas sp*.), the cofactor NAD^+ and glycine buffer with NaCl, pH 10.5. The 0.1 mM glycine-NaCl buffer was prepared by dissolving 5.85 g/l NaCl and 7.5 g/l glycine in water (MQ water) and adjusting the pH to 10.5 with NaOH. The NAD^+ stock solution was prepared from 0.6 g NAD^+ in 20 ml MQ water. The GDH solution was prepared with a concentration of 0.3 U/µl in MQ water. For the enzyme mix, 220 µl NAD^+ solution and 110 µl GDH solution were diluted with 4.66 ml NaCl-glycine buffer. The 10 mM glycerol standard solution was prepared from 18.23 mg glycerol in 2 ml YNB. 5 µl of the sample or standard solutions were placed in each well of the microtitre plate, and the reaction was started by adding 45 µl of the enzyme mixture. The reaction mixture was incubated at room temperature for 30 min before the absorbance at 340 nm was determined with the microtiter plate reader (µQuant, Biotek). Four aliquots of each sample were analyzed.

2.8. Influence of ETC-inhibitors on the cell viability

The cell vitality was determined using the tetrazolium salt WST-1, which is a redox indicator. The amount of formazan can be quantified by measuring the absorbance at the wavelength of 450 nm. The working culture of *C. albicans* was prepared in YNB (+ 2% of glucose or galactose as carbon source). ETC-inhibitors concentrations were added to result in Table 1. After cultivation time of 3h, 160 µl of the cell suspension were transferred into each well of a 96 well plate (BD Falcon, New Jersey, USA) and mixed with 20 µl of the WST-1 reagent. The plate was incubated on a plate shaker at 30 °C for 30 min. The intensity of yellow color was measured at 450 nm using the microtiter plate reader µQuant.

The inhibitory effect of ETC-inhibitors was calculated according to equation 1.

Inhibitory effect (%) = 100-(Abs_{450} treated/Abs_{450} control)*100 (equation 2)

Where: Abs_{450} treated is the absorbance of the cells treated with ETC-inhibitors, and Abs_{450} control is the absorbance the cells treated with the solvent.

3. Results

3.1. Effects of ETC-inhibitors on growth rates

The growth inhibition test is the first experiment to be performed when the activity of a new anti-microbial substance is evaluated. In this study the influence of ETC-inhibitors on growth rates was studied by cultivating the chosen yeasts in YNB supplemented with 2 % of glucose or galactose as major carbon source. Yeast cultures were treated with the most common ETC-inhibitors, namely rotenone, TTFA, antimycin A and KCN to inhibit the functions of complexes I-IV of the classical respiratory pathway and SHA to inhibit the alternative oxidative pathway (AOX) in *C. albicans*. *S. cerevisiae* was only treated with the inhibitors acting on complexes II-IV due to the lack of complex I and AOX. The effects of these compounds on the growth rates of the yeasts were strongly dependent on cultivation conditions and on the particular strain. Figure 1(A) shows the effects of the inhibitors on growth rates of *C. albicans* cultivated on glucose. There was no inhibitory effect induced by most ETC inhibitors (rotenone, TTFA, KCN and SHA). Under these conditions, the growth of *C. albicans* seems to be hardly sensitive to those inhibitors, while antimycin and myxothiazol showed a slight growth inhibitory effect.

On the other hand, when glucose was replaced by galactose, a remarkable reduction in the growth rates of *C. albicans* was observed for most inhibitors (Figure1 B). Moreover, *C. albicans* growth almost stopped after being treated with antimycin, myxothiazol and pyrrolnitrin.

In terms of *S. cerevisiae*, no reduction effect on growth rates was observed for any of the compounds when glucose was used as a carbon source, as shown in Figure 2(A). In this case, *S. cerevisiae* seems to be completely resistant to all inhibitors!

Nevertheless, *S. cerevisiae* grown on galactose as a carbon source had a slower growth rate than that grown on glucose. The yeast cells growth totally stopped when they were treated with antimycin, myxothiazol or pyrrolnitrin. On the other hand, the growth of *S. cerevisiae* was strongly inhibited by KCN; more than 50 % of growth reduction was observed. The inhibitory effect of TTFA on growth of *S. cerevisiae* was the weakest, being less than 30 %, as shown in Figure 2(B).

Growth of yeasts on 2% of glucose was maintained during the inhibition of the respiratory chain. Yeast cells produce most of their ATP by fermentation or by respiration, which can both occur on glucose medium. However, respiration is the only way to produce ATP on galactose containing media.

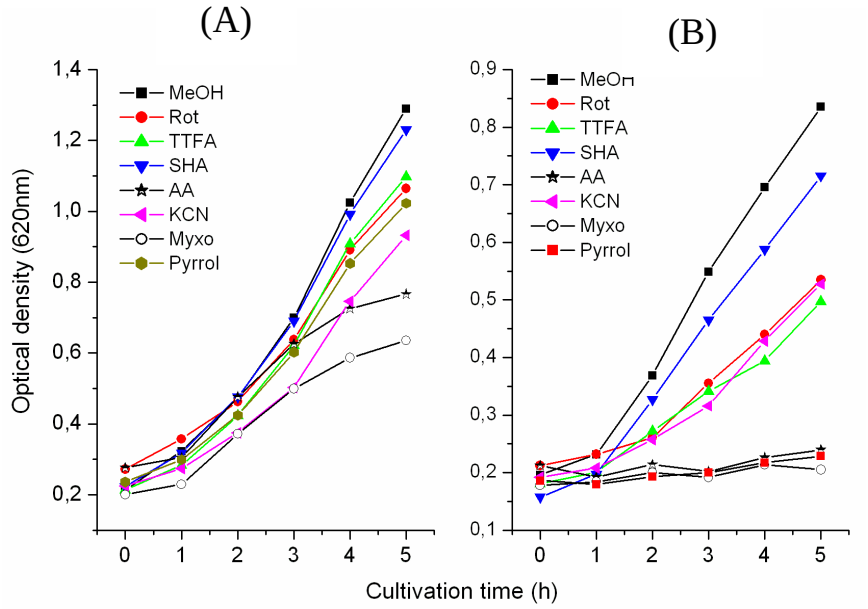


Figure 1: (A) Effect of ETC inhibitors on the growth rate of *C. albicans* in YNB (2% glucose). **(B)** Effect of ETC inhibitors on the growth rate of *C. albicans* in YNB (2% galactose). ETC inhibitor concentrations were shown in Table 1.

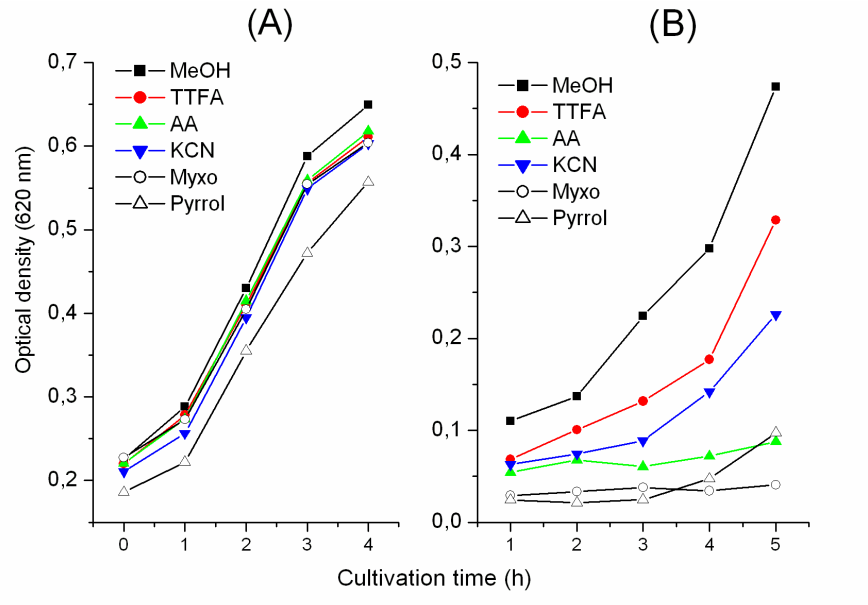


Figure 2: (A) Effect of ETC inhibitors on the growth rate of *S. cerevisiae* in YNB + 2% glucose. **(B)** Effect of ETC inhibitors on the growth rate of *S. cerevisiae* in YNB + 2% galactose. ETC inhibitor concentrations were shown in Table 2.

3.2. Effects of ETC-inhibitors on respiration

The efficacy of the respiratory chains was measured in the presence of ETC-inhibitors at different concentration ranges by measuring the oxygen uptake. As a result, although the normal oxygen uptake rate of *S. cerevisiae* was very slow, a strong sensitivity for myxothiazol and antimycin was detected. In particular, antimycin showed a blocking effect even with less than 5 ng/ml. When KCN was added to *S. cerevisiae* cultures, the oxygen consumption was reduced but its inhibitory effect was not as strong as that of antimycin or myxothiazol. However, TTFA did not exhibit a significant inhibitory effect on the oxygen uptake of *S. cerevisiae* at lower concentrations. Pyrrolnitrin strongly blocks the respiration of *S. cerevisiae* at 12 µg/ml (about 50 % of inhibitory effect), while this effect drops out with decreasing concentrations, as shown in Figure 3 (A).

For *C. albicans*, most of the available oxygen was consumed after 20 min of cultivation, as shown in Figure 3 (B), which is likely due to its high capacity of respiration. Treatment of *C. albicans* with antimycin or myxothiazol caused a dramatic decrease in the respiration process, which was much stronger than their effect on *S. cerevisiae*. TTFA impacts on the respiration of *C. albicans* are much stronger than its effects on the respiration of *S. cerevisiae*, even though still less active than antimycin and myxothiazol on both organisms. Pyrrolnitrin also shows stronger effects on *C. albicans* than *S. cerevisiae* which may reflect the importance of their mitochondrial binding sites to the respiration process.

Surprisingly, inhibition of specific electron transport pathways led to acceleration of respiration rates of *C. albicans*. This was observed after 15 min of rotenone and KCN treatment. The oxygen uptake was faster compared to the control treated cells, suggesting the involvement of alternative pathways. Because *C. albicans* has an alternative respiration pathway (AOX), detection the AOX expression was performed by RT-PCR. To this end, *C. albicans* was treated with rotenone, TTFA, antimycin and KCN as the classical respiratory chain inhibitors, in addition to SHA as AOX inhibitor. It was found that blocking the classical respiratory pathway stimulated the induction of the alternative oxidase pathway. In particular, a very strong expression of AOX was observed under KCN and antimycin treatment, as shown in Figure 4. The activation of AOX allowed *C. albicans* to maintain high rates of respiration and enhance the oxygen uptake, as shown in Figure 3(B) for rotenone (after 15 min) and KCN (after 25 min). However, the activation of the AOX pathway by antimycin or TTFA did not maintain the respiration.

We can conclude from the measurements of oxygen uptake rates of *C. albicans* and *S. cerevisiae* treated with different ETC-inhibitors that not every compound is able to block

the oxygen uptake, even if the specific binding-site is there. For example, rotenone and KCN did not show inhibitory effects on the respiration system of *C. albicans*, however, this organism encloses the complexes I and IV. In addition, the respiration response is different from one organism to the other, i.e. the response of *S. cerevisiae* towards a particular ETC-inhibitor is not similar to the response of *C. albicans* against the same inhibitor.

Therefore, measuring the growth rates or measuring the oxygen consumption rates of *C. albicans* is not adequate to figure out the mechanism or the activity of a new antifungal agent, but additional experiments are required.

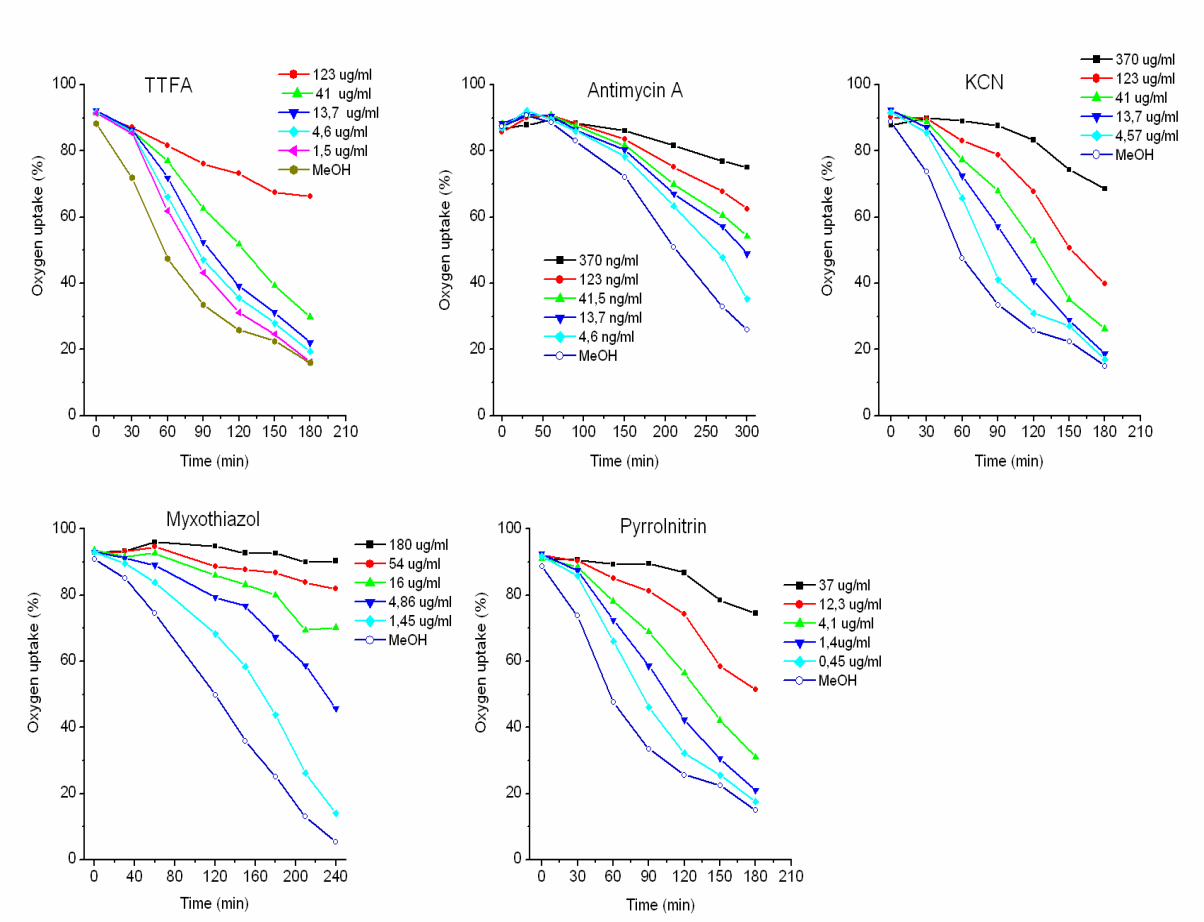


Figure 3 (A): Respiratory characteristics of *S. cerevisiae* treated with various ETC-inhibitors (TTFA, antimycin A and KCN are the inhibitors of the mitochondrial complexes II, III and IV respectively. Myxothiazol inhibits the function of complex III and pyrrolnitrin blocks the electron transfer in the early part of the respiratory chain).

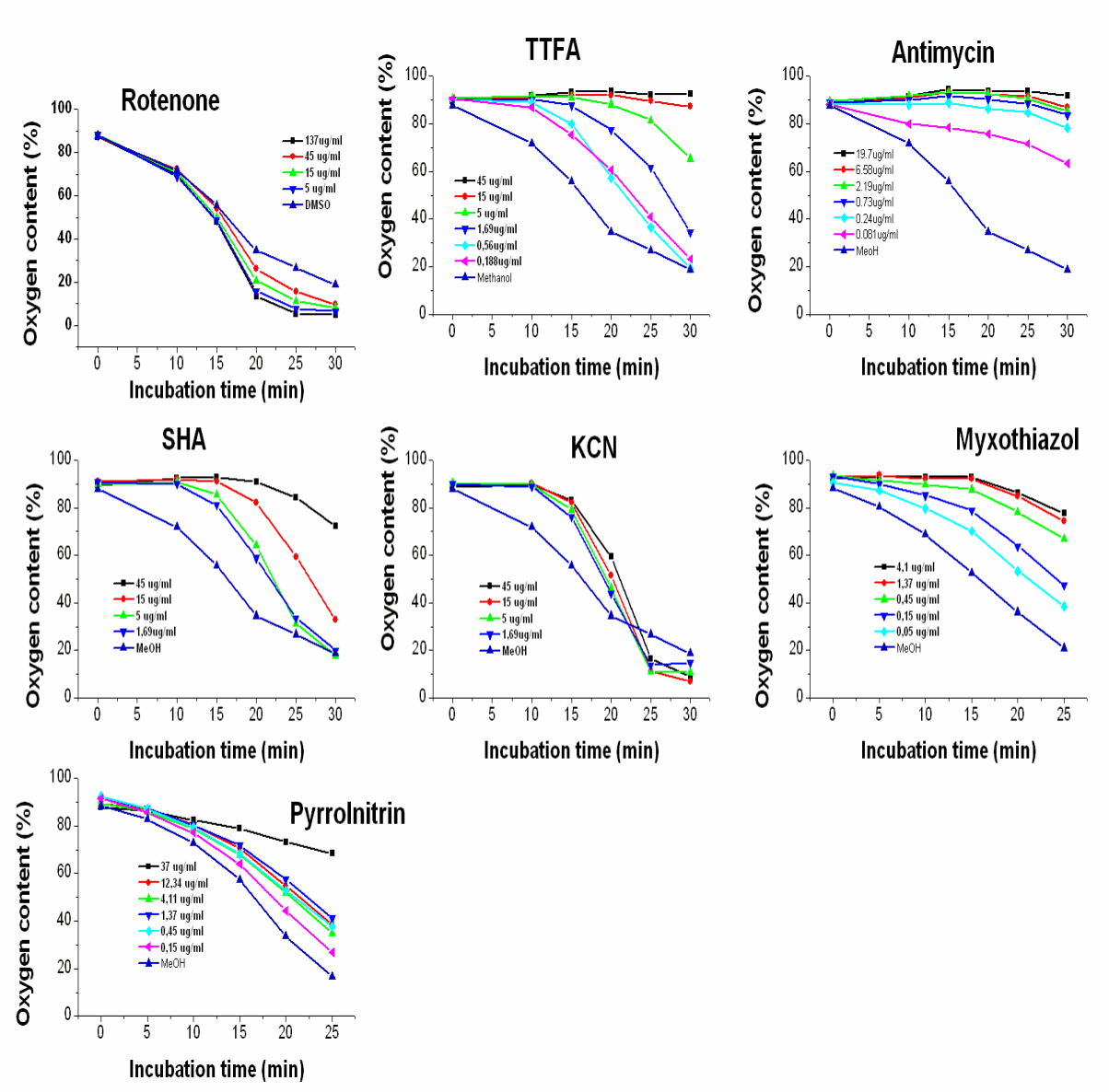


Figure 3 (B): Respiratory characteristics of *C albicans* treated with various ETC-inhibitors. (Rotenone, TTFA, antimycin A, KCN and SHA are the inhibitors of the mitochondrial complexes I, II, III and AOX, respectively. Myxothiazol inhibits the function of complex III and pyrrolnitrin blocks the electron transfer between the complexes I or II and coenzyme Q.

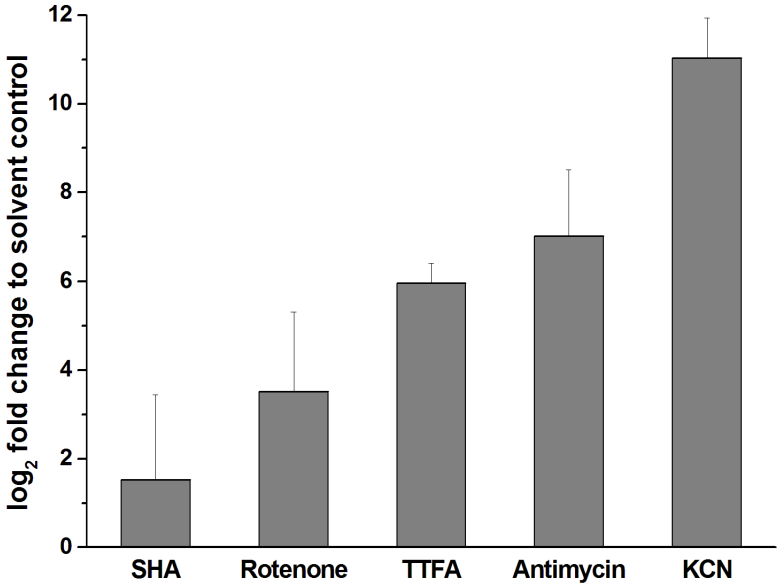


Figure 4: RT-PCR analysis of the expression of the *AOX2* gene of *C. albicans*. The averages and standard deviations of the gene expression levels relative to solvent controls in three independent experiments were calculated, and the significance of the changes in gene expression was tested by Student's t-test of normalized data ($p < 0.05$). ETC inhibitor concentrations were shown in Table 1.

3.3. Effects of respiratory inhibitors on ethanol and glycerol production

3.3.1. Glucose as carbon source

From the previous results, no strong inhibitory effects on growth rates of the chosen organisms were observed while there were effects on the oxygen uptake.

It is known that glucose can be utilized by yeasts under both aerobic and anaerobic conditions. Glucose is metabolized to pyruvate through glycolysis, and under aerobic conditions the pyruvate is incorporated into the TCA cycle. However, under anaerobic conditions pyruvate is converted to ethanol through the fermentation pathway combined with glycerol as the major by-product of fermentation. Therefore, the relationship between glucose metabolism and respiration behavior of *C. albicans* was investigated by studying the effects of ETC-inhibitors on the level of ethanol/glycerol production by yeast cells to monitor the switch between respiration and fermentation pathways. Thus, *C. albicans* was treated with the various respiratory chain inhibitors and allowed to grow in YNB supplemented with

2 % of glucose. Each concentration of respiratory chain inhibitors was selected on the basis of the entirely inhibited cellular oxygen consumption without affecting the cell growth, as described before. Figure 5 (A) shows that ethanol production increased with increasing cultivation time. The increase of ethanol production correlated with an increase in the glycerol amounts generated by *C. albicans*, as presented in Figure 5 (B). Moreover, results of the experimental assessment of the effects of ETC-inhibitors on the accumulation of ethanol and glycerol correlated to their effects on the oxygen uptake, i.e. myxothiazol and antimycin which had a strong inhibition effect on the oxygen uptake led to a significant induction of ethanol/glycerol production. No remarkable increase in ethanol/glycerol production was observed for cells treated with rotenone or KCN which had no inhibitory effect on respiration and only a slight increase in ethanol/glycerol was detected after treatment of *C. albicans* with SHA and TTFA, as shown in Figures 5 (A) and (B).

A particular feature was observed in the glycerol production of pyrrolnitrin-treated cells, where, after 4 and 5h the increase in the glycerol accumulation was significant, while the ethanol production of pyrrolnitrin-treated cells was not changed. It was reported that the HOG pathway of *Neurospora crassa* treated with pyrrolnitrin is activated which may indicate that pyrrolnitrin has an additional function beside its effect on the respiratory chain [21].

The fact that the main energy source (respiration) could be blocked or inhibited by the ETC-inhibitors without affecting the growth rate of yeasts, revealed that *C. albicans* was able to re-direct its metabolic pathways, according to the environmental changes, to compensate for the lacking energy supply. In this case the energy for survival of *C. albicans* was obtained via the fermentation pathway.

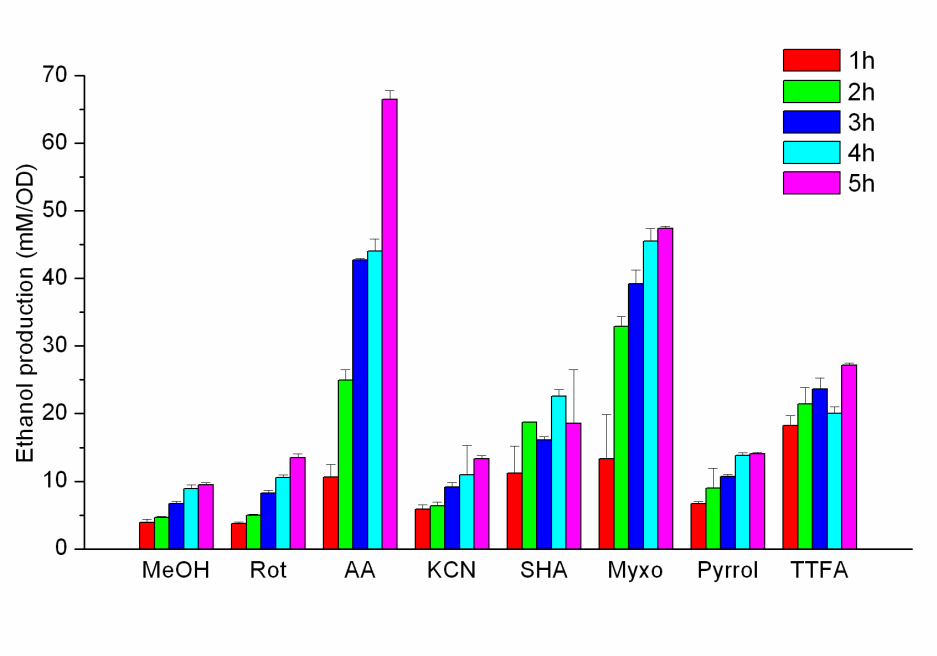


Figure 5 (A): Time course of ethanol production during *C. albicans* growth treated with ETC-inhibitors in YNB + 2 % glucose. ETC inhibitor concentrations were shown in Table 2.

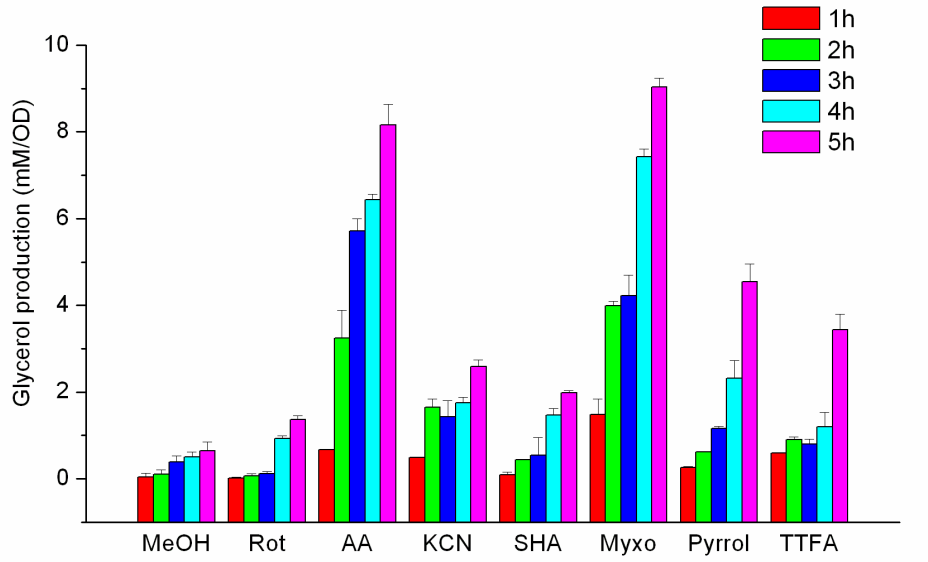


Figure 5 (B): Time course of glycerol production during *C. albicans* growth treated with ETC-inhibitors in YNB + 2 % glucose. ETC inhibitor concentrations were shown in Table 2.

3.3.2. Galactose as carbon source

Galactose is a non-fermentable carbon source, as it has to be converted in a multi-step reaction sequence to glucose-6-phosphate before it can enter the glycolytic pathway. Thus, sufficient energy is only gained from the respiration but not from fermentation and yeast cells could not grow in galactose medium in the presence of ETC-inhibitors (Figure 1, 2). This is supported by the low amounts of ethanol, which were produced by *C. albicans* growing in galactose containing medium. There was no difference between the control and the ETC-inhibitors treated cells (Figure 6).

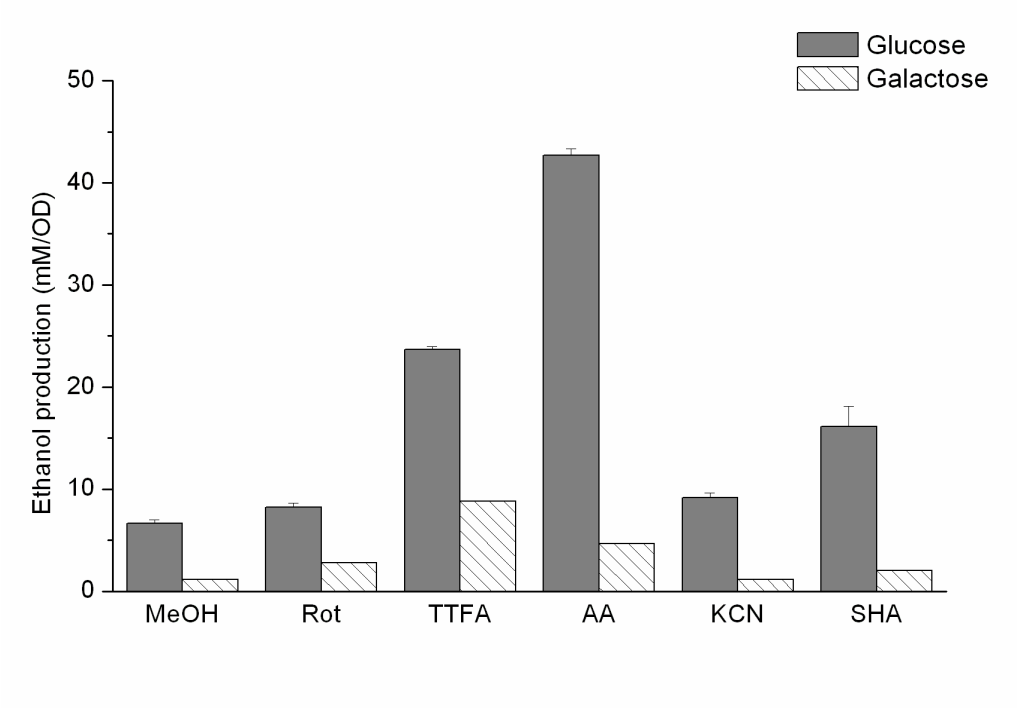


Figure 6: Shows the difference in the amount of produced ethanol by *C. albicans* grown in glucose or in galactose. The concentration of ethanol production by *C. albicans* was determined after growing for 4h in YNB + 2% glucose or in YNB + 2 % galactose and in the presence of mitochondrial inhibitors. ETC inhibitor concentrations were shown in Table 1.

3.4. Effect of ETC-inhibitors on the cell viability

The WST quantifies the cell viability based on the activity of mitochondrial dehydrogenases. These enzymes catalyze the reduction of the tetrazolium salt WST-1 to formazan by NADH via an electron shuttling compound. Thus the amount of formazan correlates to the amounts of NADH and the number of metabolically active cells.

The effects of ETC-inhibitors were dependent upon the type of carbon source used in the cultivation process. The inhibitors were more toxic in the galactose medium, particularly treatment with antimycin, myxothiazol and pyrrolnitrin, led to loss of viability of approximately 55%, as shown in Figure 7(A). In glucose-containing medium the toxic effects of inhibitors was lower, whereas the inhibition of the *C. albicans* viability was less than 25%. Similar were observed in *S. cerevisiae*, but toxic effects were generally lower than on *C. albicans*. Only KCN effects were stronger in *S. cerevisiae*, whereas more than 35 % of *S. cerevisiae* viability was inhibited by KCN, only about 15% of *C. albicans* viability was inhibited, (Figures 7).

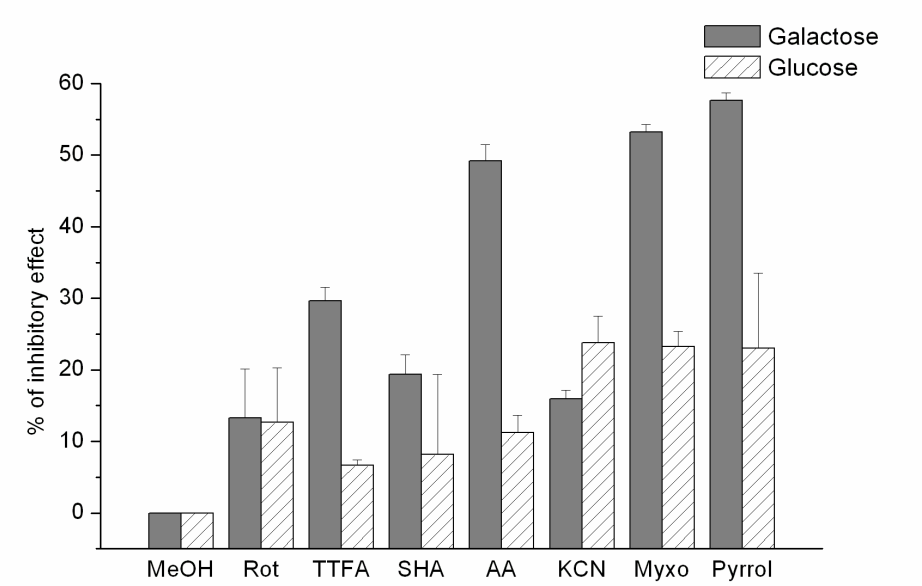


Figure 7(A): Effects of mitochondrial inhibitors on the viability of *C. albicans*. The cells were grown in YNB + 2% glucose or galactose and treated with inhibitors for 3h. Then the WST reagent was added and the color intensity was measured at 450 nm after 30 min.

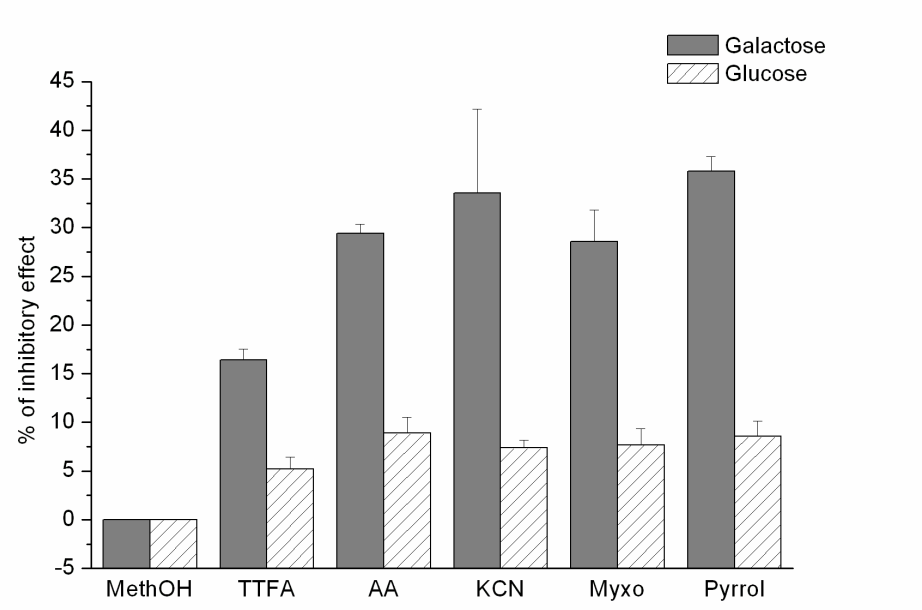


Figure 7(B): Effects of mitochondrial inhibitors on the viability of *S. cerevisiae*. The cells were grown in YNB + 2% glucose or galactose and treated with inhibitors for 3h. Then the WST reagent was added and the color intensity was measured at 450 nm after 30 min.
ETC- inhibitor concentrations in A and B were shown in Table 1.

3.5. Effects of ETC-inhibitors on the production of Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are generated in mitochondria as a normal byproduct of aerobic metabolism; 0.2–2% of O_2 consumption is estimated to be lost as superoxide under normal conditions [27]. Therefore, the involvement of the respiratory chain of *C. albicans* and *S. cerevisiae* in ROS production was investigated by measuring the induction of ROS in yeast cells after treatment with different concentrations of ETC-inhibitors. Both *C. albicans* and *S. cerevisiae* were able to produce significant amounts of ROS after incubating with specific mitochondrial inhibitors (Figure 8).

For *S. cerevisiae,* a significant increase in the ROS production was observed when the cells were treated with antimycin and myxothiazol over a wide range of concentrations. Antimycin showed a high effectively, as the amount of ROS was increased to 180% at 50 ng/ml of antimycin. Effects of KCN and pyrrolnitrin on the formation of ROS were also concentration dependent, occurring only at high concentrations, while, at lower concentrations only a slight increase in the ROS was observed. The inhibition of complex II by TTFA did not contribute to ROS production at any concentration, even at very high concentrations (740 µg/ml), as shown in Figure 8(A).

In *C. albicans*, the impacts of ETC-inhibitors on the cellular ROS production correlated to their effects on *S. cerevisiae*, as shown in Figure 8(B), while amounts of ROS generated by *C. albicans* were higher than that of *S. cerevisiae*.

In more detail, inhibition of complex I by rotenone led to a strong increase of ROS formation, the same was observed after blocking the function of complex III by antimycin or myxothiazol. In contrast, disturbance of the electron transfer by TTFA did not increase the ROS production. Blocking the alternative oxidative pathway by SHA resulted in a slight increase of ROS production.

Surprisingly, the ROS formation of *C. albicans* decreased after treatment with KCN by about 40 %, whereas concentrations below 1.3 µg/ml KCN had no effect. This may imply that the activation of the AOX pathway by higher concentrations of KCN protected the cells from potential damaging effects caused by KCN and led to a decrease of ROS.

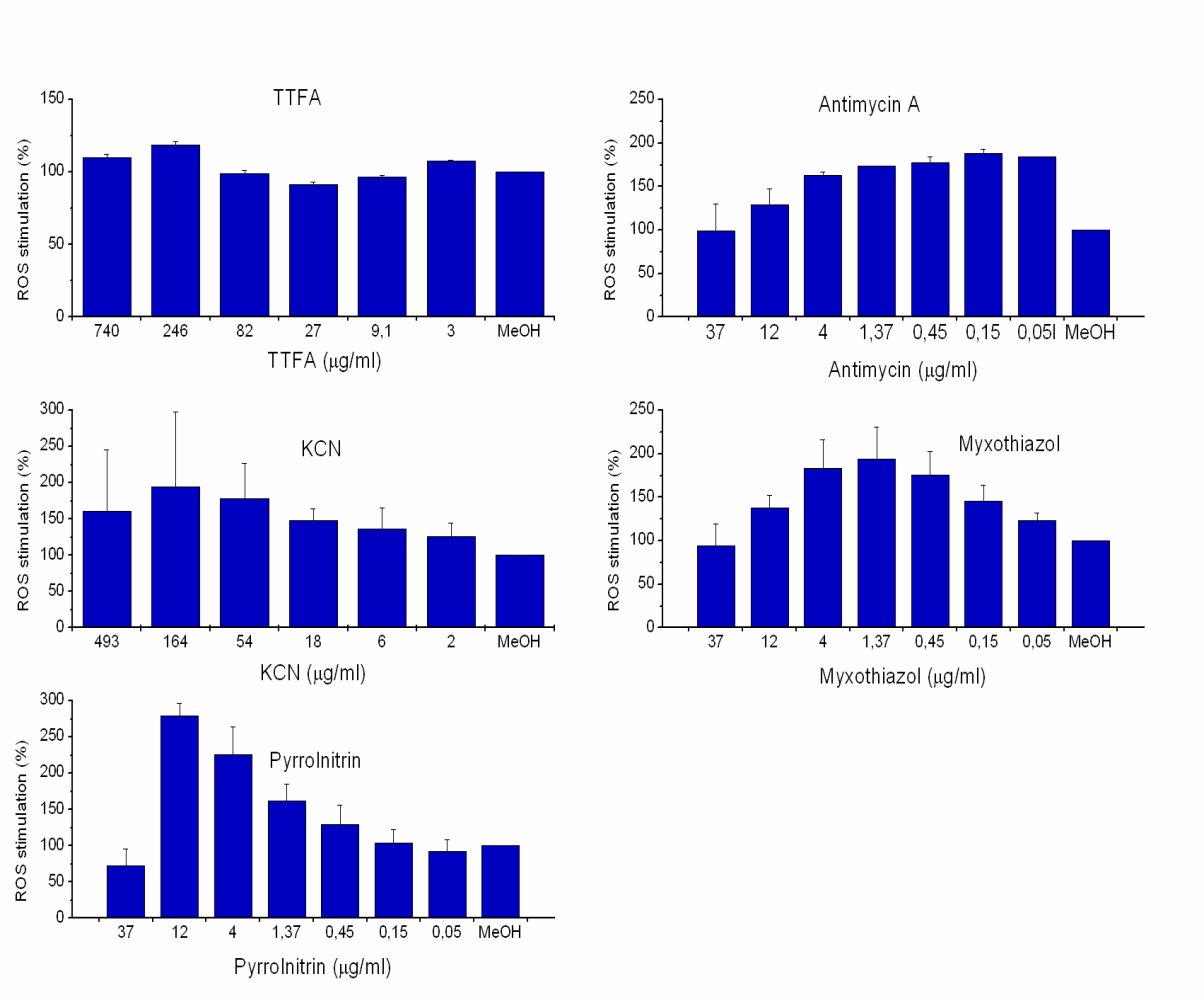


Figure 8 (A): Effects of the mitochondrial inhibitors on the stimulation of ROS production of *S. cerevisiae* after incubation with ETC-inhibitors at 30 °C in a 96-well plate for 2h.

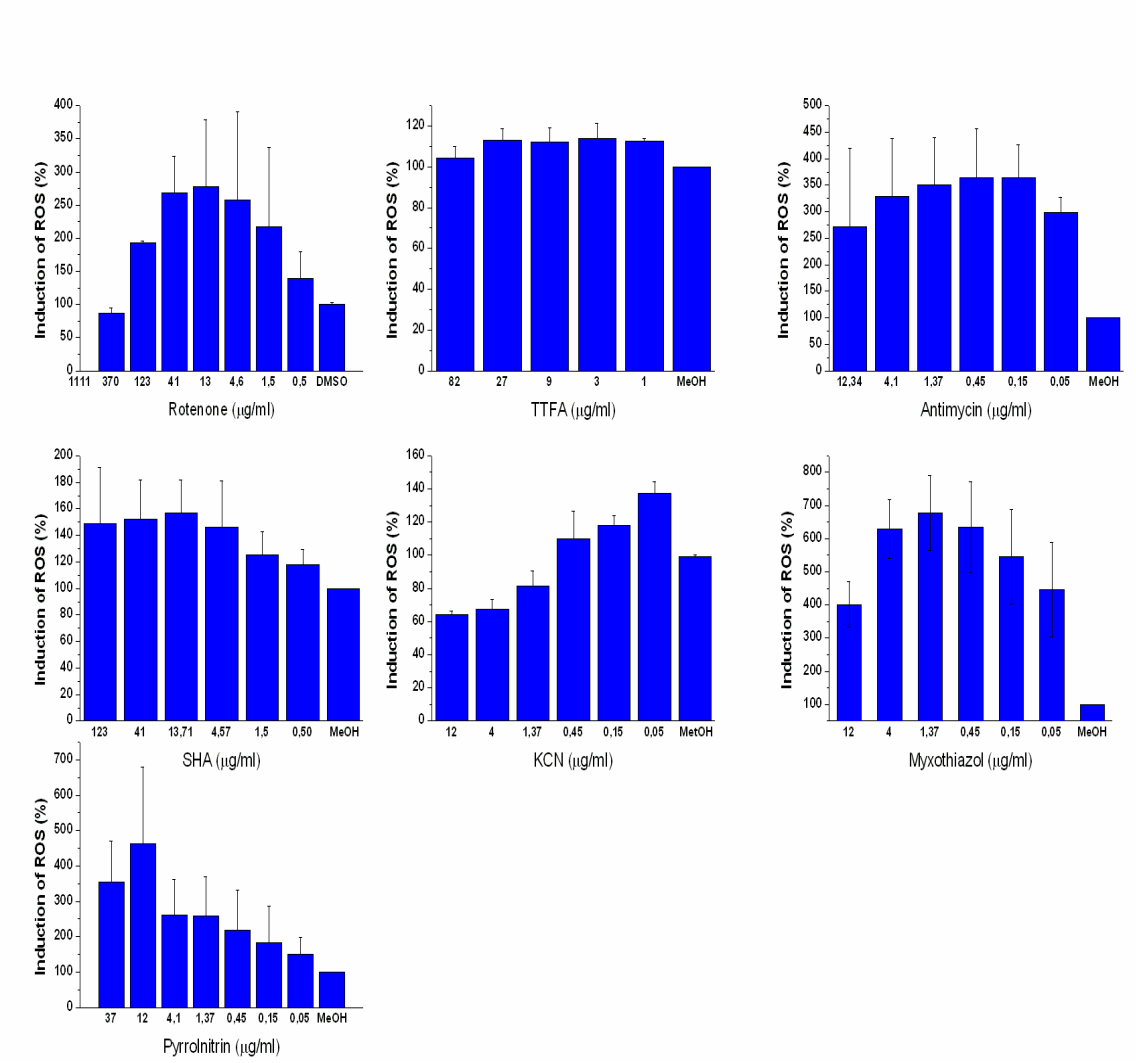


Figure 8 (B): Effects of the mitochondrial inhibitors on the stimulation of ROS production of *C. albicans* after incubation with ETC-inhibitors at 30 °C in a 96-well plate for 2h.

4. Discussion

When *C. albicans* and *S. cerevisiae* were exposed to ETC-inhibitors and glucose was the carbon source in the cultivation medium, no or only slight growth defects were observed. Nevertheless, by replacing glucose with galactose, a significant growth inhibition was found in *C. albicans* and *S. cerevisiae* after treatment with the respiratory chain inhibitors. These results were almost identical with the results of the cell viability assay, where the degree of inhibitors toxicity was dependent on the type of carbon source utilized by the yeast cells during the cultivation process where dramatic toxicity effects were found for most of the inhibitors on *C. albicans* and *S. cerevisiae* when galactose was the carbon source. The reason for cell survival after the treatment with respiratory chain inhibitors in presence of glucose could be explained by the activation of the fermentation pathway. Blocking cellular respiration leads decreasing energy production per carbon source. The switch fermentation was necessary delivers sufficient energy for cell survival due to the high reaction rates.

The significant increase in the ethanol/glycerol production by the treated cells growing on glucose proved that the cells were enforced to change the direction of the metabolic pathway from the respiration to the fermentation. However, galactose was not an easily fermentable sugar; therefore, cells which have a reduction in the oxygen uptake due to the effect of the ETC-inhibitors could not grow.

The direct inhibitory effect of the respiratory blockers was investigated by measuring the oxygen uptake rate and the capability of mitochondrial complexes to produce reactive oxygen species (ROS). Concerning the ROS results, the major formation of ROS by *C. albicans* and *S. cerevisiae* after addition of respiratory chain inhibitors, in particular rotenone, antimycin or myxothiazol, revealed that there were two main sources for the mitochondrial ROS production, NADH-dehydrogenase and cytochrome *c* reductase [28, 29]. In contrast, KCN-treated *C. albicans* produces a lower ROS amount than the untreated-*C. albicans*, whereas, KCN led to slight increase in ROS production in *S. cerevisiae*.

This indicates that the AOX in *C. albicans* plays a role in the detoxification of superoxide formation (ROS defense systems) caused by the dysfunction of the mitochondrial complex IV. The results of pyrrolnitrin effects on the ROS production in both *C. albicans* and *S. cerevisiae* refer to the biding-site of pyrrolnitrin close to complex I.

Concerning the effects of the ETC-inhibitors on the O_2 consumption the results showed that most inhibitors blocked the O_2 uptake. However, rotenone and KCN increased the O_2 uptake

rate of *C. albicans*. This effect was not found in *S. cerevisiae*, where all of the respiratory chain inhibitors reduced the O_2 uptake.

The increase in the O_2 consumption after addition of rotenone or KCN to the cultivation medium could be explained by the presence of three respiratory pathways for the oxygen uptake in *C. albicans*, as shown in Scheme 1. Moreover, the alternative oxidative pathway is induced by blocking the function of the classical respiration [30]. In order to confirm the activation of this pathway, the RT-PCR experiment was performed and the results confirmed this postulation. Inhibition of any of the classical respiratory chain led to the induction and activation of the AOX, and hence, the electrons passed directly from the coenzyme Q to the AOX and the oxygen was reduced to H_2O.

5. Summary and conclusion

There is more than one reason to consider the respiratory chains of *C. albicans* as potential drug targets. These respiratory pathways have significant impacts on the virulence factors and moreover one of their main components, the AOX, is not found in the human cells. The respiratory chain is not essential for growth in glucose-rich medium, and due to the presence of several terminal oxidases even inhibition of oxygen uptake by specific inhibitor can be bypassed. Therefore, the current study provided strategy consisting of several biochemical assays to record different biological responses of *C. albicans* after treatment with different inhibitors, which make this strategy valid, reliable and worthy applying in the mechanistic investigations and understanding the mode of action of the respiratory chains drug targets.

Table 3 summarizes the obtained results of ETC-inhibitors effects on different aspects of *S. cerevisiae*. Rotenone was not applied to *S. cerevisiae* due to the lack of complex I and thus is not included in the Table 3. In table 4 the corresponding data from *C. albicans* are collected.

Table 3: Collection of the obtained findings of the effects of ETC-inhibitors on *S. cerevisiae* YNB was the basis of the cultivation medium in all experiment except in the measuring of oxygen uptake experiment it was YPD

	Effect on growth Inhibition (%)		**Blocking of O_2 Uptake (%)**	**Inhibition of cell viability (%)**		**ROS (%)**	**Ethanol/ Glycerol Production (%)**
2% of carbon source	**Glucose**	**Galactose**	**YPD Glucose**	**Glucose**	**Galactose**	**MQ-H_2O**	**Glucose**
TTFA	6	25	27	5	15	107	199/141
Antimycin	5	41	63	10	30	180	187/190
KCN	7	31	17	10	32	150	149/181
Myxothiazol	6	40	53	10	30	194	230/165
Pyrrolnitrin	20	40	65	10	35	270	173/136

Table 4: Summary of the results of ETC-inhibitors effects on *C. albicans*, n.d. represents the missing experiments of this study (not determined).

	Effect on growth Inhibition (%)		**Blocking of O_2 Uptake (%)**	**Inhibition of cell viability (%)**		**ROS (%)**	**AOX**	**Ethanol/ Glycerol Production (%)**	
2% of carbon source	**Glucose**	**Galactose**	**YPD Glucose**	**Glucose**	**Galactose**	MQ-H2O	**YPD Glucose**	**Glucose**	**Galactose**
Rotenone	9	55	(-32)	12	12	260	3	123/184	**No effect**
TTFA	11	32	60	8	30	111	6	355/236	
Antimycin	50	97	62	12	50	350	7	641/1270	
KCN	30	52	25	25	15	81	11	241/346	
Myxothiazol	48	93	58	25	53	650	n.d.	589/1470	
Pyrrolnitrin	14	94	39	25	57	450	n.n	160/460	
SHA	2	14	57	10	20	150	1.5	137/290	

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Self-mediated electron transfer from *Candida albicans* to electrodes: Investigation of the classical respiratory pathway

Abstract

Electron transfer processes from microorganisms to electrodes, can be exploited in diagnostic tools or in microbial fuel cells. Even eukaryotic microorganisms transfer electrons to electrodes without the supplement of exogenous mediators. As the origin of these processes is poorly understood, we have studied the transfer of electrons from the fungi *Candida albicans* and *Saccharomyces cerevisiae* to carbon paste electrodes. Cyclic voltammograms (CV) showed irreversible oxidation currents in the potential range from 700 – 800 mV produced by viable yeasts. The peak current linearly increased with yeast cell numbers. By electrochemical and mass spectrometric analysis of the electrolyte solution, we identified tryptophol as the electron-transferring compound. This is the first report of a fungal self-mediated electron transfer process and of the function of aromatic alcohols as fungal electron mediators. Tryptophol concentrations increased with tryptophan concentration in the cultivation medium; this effect was correlated with increasing electrochemical signals. We also observed strong involvement of the respiratory chain in tryptophol production, as signals increased with the respiratory metabolic activity of the cells. Moreover, the addition of most electron transport chain inhibitors reduced the signal, but the inhibitor of the alternative oxidase AOX, salicylhydroxamic acid, led to a significant increase, suggesting an involvement of the classical respiratory chain, with cytochrome c oxidase (COX) as a relevant protein complex. We therefore additionally used single gene deletion mutants of nuclear encoded COX subunits of *S. cerevisiae*. All of these mutants showed reduced electrochemical signals, i.e., tryptophol concentrations; the deletion of subunit COX5a almost completely abolished the signal.

Keywords*:* electrochemical reaction of yeasts; electron transfer inhibitors; cytochrome c oxidase; tryptophol

1. Introduction

Microorganisms use the oxidation of a large variety of substrates to gain energy and thus have to transfer electrons to suitable electron acceptors. Examples of natural electron acceptors are oxygen, pyruvate, nitrate, and even metal oxides depending on the availability of oxygen and the capability of anaerobic respiration. In recent years, interest in electron transfer processes in particular from microorganisms to solid materials has increased, as these processes can be exploited technologically for the development of microbial fuel cells [1] or diagnostic tools [2]. In these systems, electrons are transferred from microorganisms to solid electrodes with (for example, [2-4]) or without [5, 6] the addition of artificial electron acceptors (mediators) leading to the generation of electrical currents. Mediatorless electrochemical sensor systems for the detection of microorganisms have been described since the late 1970s [5, 7]. Various bacteria and the yeast *Saccharomyces cerevisiae* have been included in such studies [6], and oxidation peaks have been observed in the potential range from +600 to +800 mV (vs. Ag/AgCl reference electrodes) for all organisms.

Faradaic current densities are dependent on the concentrations of electron transfer agents and on the efficiency of electron transfer. Whereas the first are influenced by properties of the cell culture, such as cell density or metabolic activity, the latter depends on the electrochemical properties of the electron transferring compound and on its steric accessibility, i.e., the physical contact between the respective redox center and the electrode. As the oxidation of carbon sources is the major source of electrons, the most important electron transfer system in organisms is the electron transport chain linked to aerobic or anaerobic respiration. In bacteria, the respective proteins and electron shuttles are located in the cytoplasmic plasma membrane, whereas in eukaryotic cells, they are present, in particular, in the mitochondria.

Nearly all microorganisms cover with the cytoplasmic membranes with additional layers, such as cell walls, peptidoglycan or outer membrane, so that not only proteins in mitochondria but also in cytoplasmic membrane are physically separated from the extracellular environment. Electrons which are transferred to extracellular electron acceptor, such as electrodes in bio-electrochemical systems or metal oxides in the natural environment, have to cross these barriers. In bacteria the respective electron transfer strategies are extensively investigated. The involvement of periplasmic c-type cytochromes [8-10], the presence of conductive nanowires in the outer membrane [11, 12] and secreted shuttles compounds as electron carriers such as phenazines [13, 14] , flavins [15, 16] and quinines [17] was shown. Thus, most reports of microbial fuel cells deal with the development of

several bacterial systems [18]. However, the use of yeasts has also been suggested [1] as they can grow on cheap substrates and do not require anaerobic conditions. Nevertheless, information on the underlying electron transport mechanisms in yeasts, i.e., on the identity of the electron transfer agent, is still limited. The existence of membrane-bound shuttling compounds [1] has been proposed. These could be either proteins [19] or cofactors, such as cytochromes [6] or CoA [7]. However, unambiguous proof involving the identification of the respective compound is still missing.

In our study, we have focused on the opportunistic yeast *Candida albicans*, as a need exists for rapid and simple diagnostic tools [2], which might be achieved by a mediatorless electrochemical system. Moreover, *C. albicans* offers an opportunity to extend the study of parameters influencing the electron transfer mechanisms to electrodes, as the structure of the respiratory chain is significantly different from that of mammalian cells or of the baker´s yeast, *S. cerevisiae* (Fig. 1) [20]. *C. albicans* has three respiratory pathways: In addition to the classical respiratory chain (CRC), an alternative respiratory pathway [21-23] and a parallel electron transport chain (PAR) [24] were described. The classical respiratory chain comprises all four enzymatic complexes in particular proton pumping complexes NADH dehydrogenase, also known as NADH-ubiquinone oxidoreductase, (complex I), ubiquinol-cytochrome c oxidoreductase (complex III), and cytochrome c oxidase (complex IV) as the terminal electron donor, where oxygen is reduced.

In *S. cerevisiae*, complex I is replaced by three NADH dehydrogenases [25, 26], which are located on the external and the internal sides of the mitochondrial membrane and which do not have proton-pumping capabilities.

The alternative respiratory pathway is generally conferred by a cyanide-insensitive alternative oxidase located on the matrix side of the inner mitochondrial membrane and encoded by two nuclear genes, *AOX1* and *AOX2*. *AOX1* is constitutively expressed, whereas expression of *AOX2* is induced, for example by the presence of inhibitors of complexes of the CRC. These alternative oxidases catalyze the direct oxidation of ubiquinol by oxygen without proton translocation and, thus, bypass complexes III and IV and enable respiration even in the presence of downstream CRC inhibitors. Due to the lack of a proton gradient energy storage from NADH oxidation is less efficient than in the CRC. The PAR is only activated when both the CRC and the AOX pathways are totally blocked, allowing electron flux to be redirected upstream of complex III, in parallel to the CRC [24].

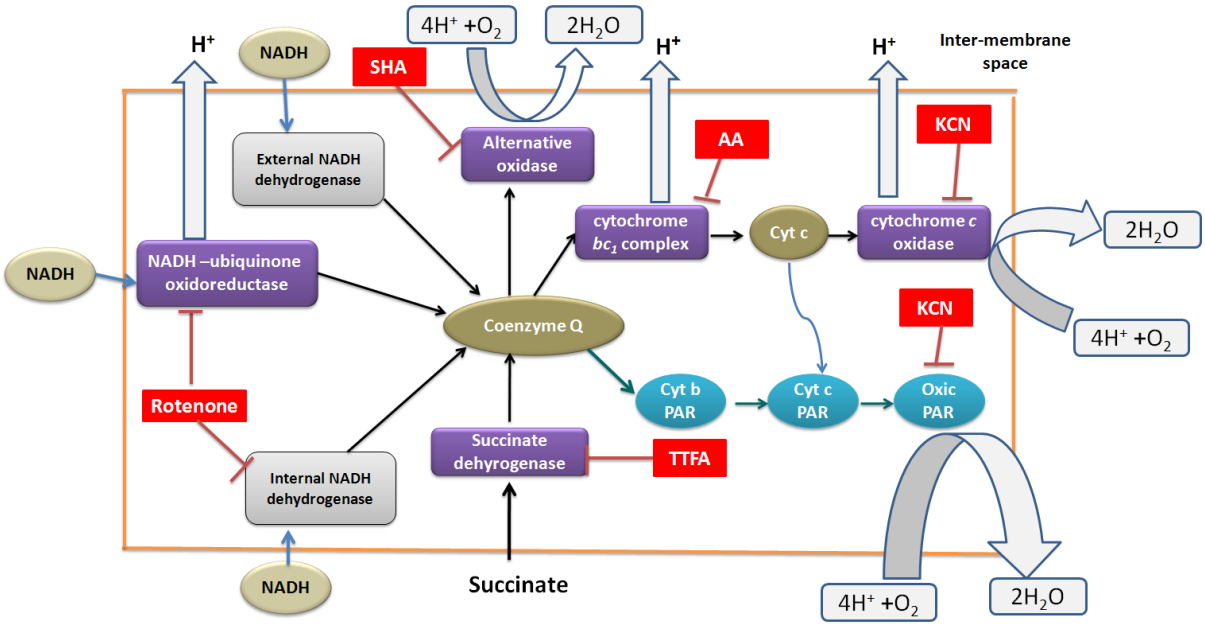

Figure 1: Respiratory chain structure of *C. albicans.* NADH is oxidized by any of the NADH-dehydrogenases leading to reduced coenzyme Q. This is also produced from the oxidation of succinate by succinate-dehydrogenase. Reduced coenzyme Q is oxidized again either by oxygen through the alternative oxidase, or by cytochrome c in the cytochrome bc_1 complex. Cytochrome *c* is finally oxidized by oxygen by cytochrome *c* oxidase. These latter reactions can also be catalyzed by components of the parallel pathway PAR.

In this study, we have studied the influence of the metabolic activity and of the activity of single complexes of the respiratory chain on the transfer of electrons from the yeasts *C. albicans* and *S. cerevisiae* to carbon paste electrodes. For both yeasts, we have obtained oxidative peak currents that lie in the same potential range as described earlier for other microorganisms [6] and that correlated with cell numbers and the activity of the classical respiratory chain. Inhibition of the alternative oxidase (AOX) led to an increase of signals. Obviously, the fraction of the total number of electrons, which is transferred from NADH to oxygen via this bypass pathway, was re-directed to the classical respiratory chain and increased the electrochemical signals. Voltammograms, which were obtained from the electrolyte solution from which the suspended yeast cells had been removed, showed the same oxidation peaks as the cell suspension. Thus, electron transfer was independent of the presence of the cells, i.e., was attributable to a secreted compound. Further analysis led to the

identification of tryptophol as electron transfer shuttle. This is the first report establishing the capability of fungi to produce an endogenous soluble electron transfer mediator.

2. Materials and methods

2.1. Materials and instruments

Potassium phthalate mono basic was purchased from Riedel-de Haen, and a 100 mM solution was adjusted to pH 7 by KOH. YPD broth, YNB without amino acids, tryptophan, antimycin A from *Streptomyces sp*., and rotenone were purchased from Sigma. YPgal medium contained yeast nitrogen base with amino acids from Sigma, peptone from Roth, and galactose from Merck. 2-(4-Hydroxyphenyl) ethanol (tyrosol) was purchased from Fluka. Salicylhydroxamic acid (SHA), farnesol, and phenylethyl alcohol were from Sigma-Aldrich, and tryptophol was from Aldrich. Synthetic carbon powder was obtained from Sigma-Aldrich and paraffin oil from Fluka.

All OD measurements were performed in 180 µl sample volumes with the microtiter plate reader µQuant (BioTek Instruments GmbH, Bad Friedrichshall, Germany), and fluorescence was determined with the microtiter plate reader Cytofluor, Reader Series 4000 (PerSeptive Biosystems, Framingham, USA). The electrochemical measurements were carried out with a computer-controlled Gamry Potentiostat / Galvanostat / ZRA G750 (Gamry, Pennsylvania, USA)**,** which was connected to a three-electrode electrochemical cell with a carbon paste working electrode, a Pt disc auxiliary electrode, and a KCl saturated Ag/AgCl reference electrode.

2.2. Preparation of yeast samples

2.2.1 Standard cultivation conditions

The following fungal strains were used: *Candida albicans* strains CAF2-1 and *Saccharomyces cerevisiae* BY4741 and its single gene deletion mutants *COX5a, COX5b, COX7, COX8,* and *COX12* (Euroscarf, Frankfurt, Germany). Yeasts were cultivated overnight in 250 ml flasks with 50 ml YPD medium at 30 °C. A pre-culture was prepared by diluting the overnight culture to an optical density (OD_{620}) of 0.2 in 25 ml YPD. The yeasts were allowed to grow for 3 hours so that they reached the exponential growth phase. The working culture was prepared by diluting the pre-culture to an OD_{620} of 0.l in YPD, if no other conditions are mentioned. After cultivation for another 3 hours, the OD_{620} was

recorded. The cells were harvested by centrifugation (Eppendorf centrifuge 5804R) at 5000 rpm at room temperature for 5 min and resuspended in 1 ml PBS.

2.2.2 Killing of microorganisms

Non-living cells of *C. albicans* were obtained from the working culture after the cultivation time of 3 h by autoclaving at 121 °C for 20 min. The cells were harvested and resuspended as described above.

2.2.3 Electron transfer chain inhibitors

Microorganisms were treated with the electron transport chain (ETC) inhibitors using the final concentrations given in Tab. 2. The concentrations of each compound were chosen for each compound on the basis of the inhibitory effects on oxygen consumption (Chapter 2). The inhibitors were added to the medium of the working culture, and the yeasts were allowed to grow in the presence of the inhibitors at 30 °C for 3 h and were harvested and resuspended as described above.

2.2.4 Effect of tryptophan

The working culture was prepared from YNB medium without amino acids supplemented with 2% glucose and 0 mg/l, 20mg/l, or 100 mg/l tryptophan. *C. albicans* was cultivated for 3 hours and harvested as described before.

2.3. Preparation of carbon paste electrodes and voltammetric procedure

1 g synthetic carbon powder (1-2 micrometers) was thoroughly mixed with 0.4 ml paraffin oil. A portion of the paste was packed into the tip of the electrode assembly with an electrode area of 0.5 cm^2. When the working electrode had to be regenerated or cleaned, a small part of the paste was cut off and polished with wetted filter paper. Before use, the working electrode was activated in 0.1 M phthalate buffer pH 7 by cyclic scans from 0 - 1 V with scan rates of 50 mV/s. 1 ml of a microbial suspension in PBS was introduced into the electrochemical cell, which contained 24 ml 0.1 M phthalate buffer (pH 7). The cells were mixed with the buffer and allowed to adapt for approximately 5 min. Linear sweep voltammograms (LSV) and cyclic voltammograms (CV) were recorded in the potential range of 0 – 1 V with scan rates of 50 mV/s. In the figures, the first voltammogram of a series is shown. It is representative for at least three independent repetitions of the experiments. All electrochemical experiments were carried out without stirring at room temperature.

For metabolic activation of *C. albicans* and *S. cerevisiae*, the phthalate buffer was supplemented with 20 g/l glucose or galactose. 1 ml of the microbial suspension of *C. albicans* or *S. cerevisiae* in PBS were incubated in 24 ml of these buffer solutions containing either carbon source for 30 min at 30 °C before the electrochemical measurement was performed.

2.4. Oxygen determination and viability tests

Oxygen consumption was monitored during the cultivation of *S. cerevisiae* in YPgal medium instead of YPD medium to avoid fermentative metabolism. Oxygen was determined with round-bottomed OxoPlates® (PreSens, Regensburg, Germany) following the procedure given by the manufacturer. Briefly, the fluorescence of the indicator and the reference dye were determined from the bottom of the plates at the excitation wavelength λex of 530 nm and the emission wavelength λem of 620 nm for the indicator dye and λem 590 nm for the reference dye. Calibration was performed with water saturated with air (100%) and with an aqueous Na_2SO_3 solution (10 g/l) (0%) according to the supplier's protocol (32).

Cell viability was determined with the WST assay (WST-1: [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium]) (Roche). 10 µl of the WST reagent was added to each well of a 96-well microtiter plate containing 170 µl of a yeast cell suspension in the culture medium. The reagent was allowed to react for 20 min, before the absorbance was measured at 450 nm.

3. Results and discussion

3.1 Electron transfer reaction from *C. albicans*

3.1.1 Viable yeasts produced anodic peak currents

Cyclic voltammograms (CV) of *Candida albicans* suspension showed a clear anodic peak at potentials of 600 – 800 mV and no corresponding cathodic peak (Fig. 2a), indicating an irreversible electrochemical oxidation reaction, as previously described for other microorganisms [5-7]. Thus, the following studies focused on the anodic reaction, and mainly linear sweep voltammograms (LSV) were recorded, as shown for cell suspensions of *C. albicans* and *S. cerevisiae* in fig. 2b. For both organisms, an anodic peak appeared between 700 and 800 mV. However, when the cells were autoclaved before they were injected into the electrochemical cell, no electrochemical response was observed. Thus, the anodic current was obviously an indicator of viable organisms. As the anodic peak potentials (*Ep*) were in the same potential range for both yeasts, we assumed that the same electron shuttle was responsible for this response.

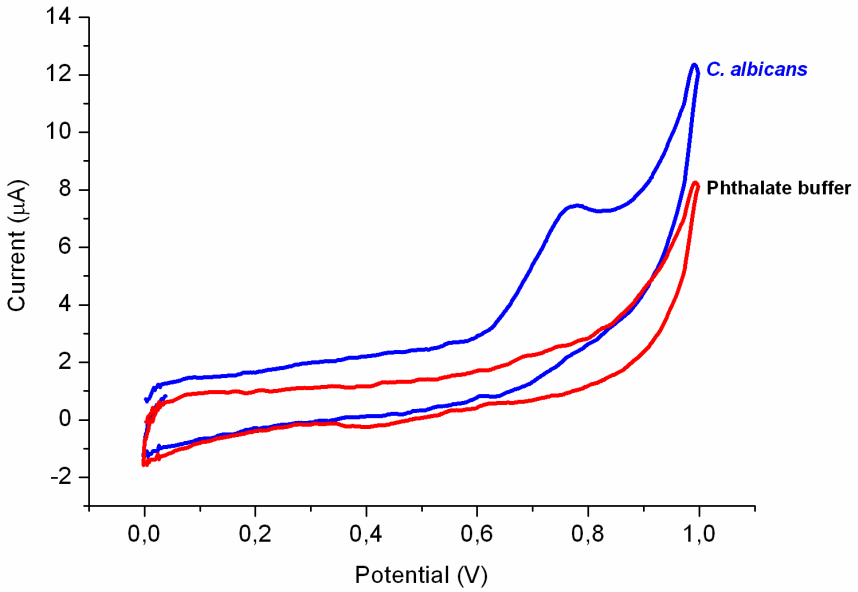

Figure 2a: Cyclic voltammogram of *C. albicans* in comparison with the blank phthalate buffer (scan rate: 50 mVs^{-1}, final concentration of cells in the electrolyte: 3.8 x 10^6/ml).

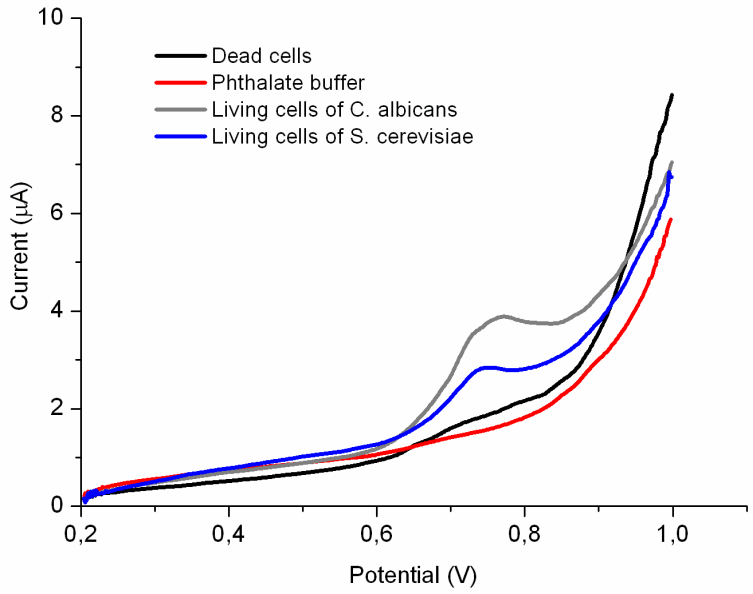

Figure 2b: Linear sweep voltammograms of *C. albicans*, *S. cerevisiae*, and dead cells of *C. albicans* with cell counts of 3.8 x 10^6/ml, 9.15 x 10^6/ml, and 2.2 x 10^6/ml, respectively.

3.1.2 Composition of electrolyte solutions influenced peak currents

The influence of the buffer composition on the oxidation current was studied. Electrolyte solutions, such as phosphate-buffered saline (PBS), Britton-Robinson buffer (pH 7), phthalate buffer pH 7 (0.1M), phthalic acid solution (0.1M) pH 1.9, and KCl pH 5.5 (0.1M), were used. In all solutions, the anodic peak was obtained for both yeasts. However, potassium phthalate buffer resulted in the highest peak current (Tab. 1) and was chosen for the following investigations.

Microbial viability and activity of enzymes are strongly dependent on pH; therefore, the influence of the pH was investigated in the range from pH 4 to pH 8. The anodic current increased with increasing pH up to pH 7. At higher pH the peak current decreased again (Fig. 3). Therefore, pH 7 was chosen for the following investigations.

Table 1: Effect of electrolyte composition on the oxidation peak current of *C. albicans*

Electrolyte	Current (µA)	Potential (mV)	pH
K-phthalate	3.6	745	7
PBS	1.9	751	7
Phthalic acid	1.62	701	1.9
Britton-Robinson	2.3	737	7
KCl	1.89	723	5.5

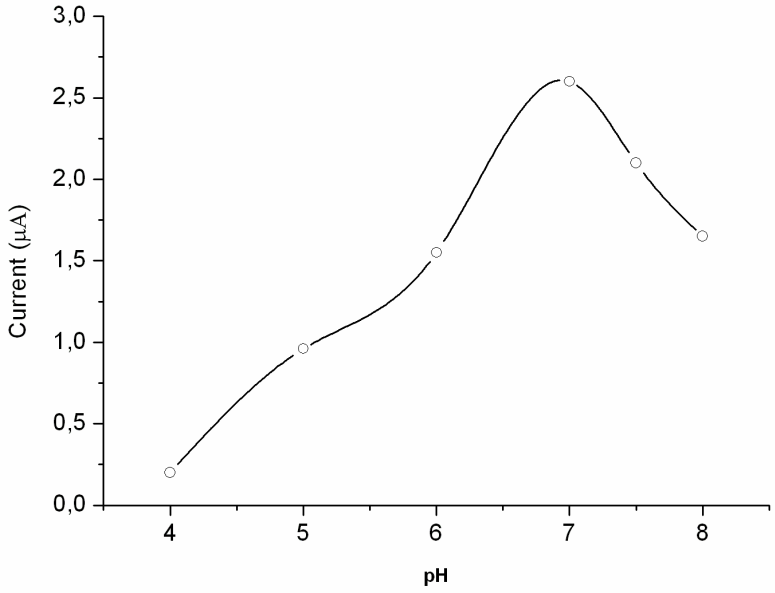

Figure 3: Effect of pH on the oxidation current of *C. albicans* at OD_{620} 0.5. (Cell number of *C. albicans* (3.8×10^6/ml))

3.1.3 The peak current correlated with cell numbers

As shown in fig. 4a, a linear increase of the peak current with the number of *C. albicans* cells was found in the range from 1.57 x 10^5 to 1.3 x 10^7 cells/ml. The signals were recorded during *C. albicans* cultivations (Fig. 4b), and good correlations to growth curves resulting from the WST viability test and OD_{620} nm measurements were obtained.

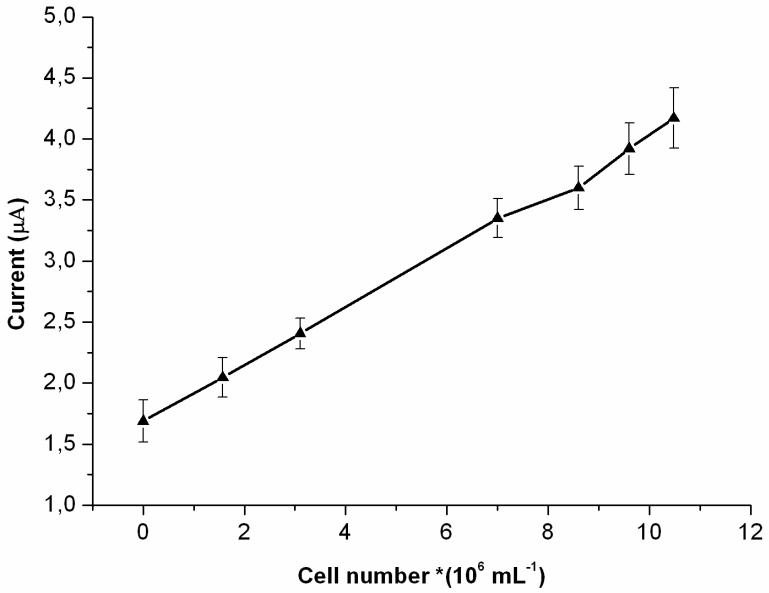

Figure 4a: Relationship between cell numbers of *C. albicans* and the anodic peak current in LSVs by using scan rates of 50 mV/s.

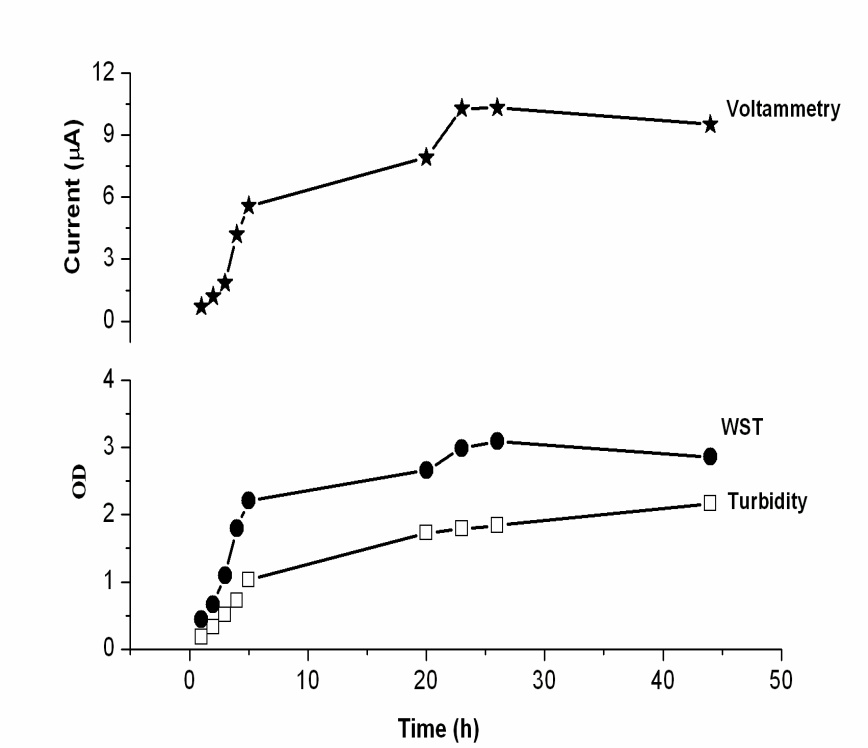

Figure 4b: Growth curve of *C. albicans* determined by OD_{620} nm (turbidity), a viability test (WST) (OD_{450} nm), and the electrochemical method (current in LSV). *C. albicans* was cultivated in YPD following standard cultivation conditions (2.2.1). However, the working culture was not halted after 3 h but allowed to continue for 45 h.

3.2 Identification of extracellular electron shuttles

3.2.1. Electrochemical activity of the supernatant

Although the electron transfer capability of yeasts without exogenous mediators has frequently been reported [1, 5, 7], the mechanism is still unknown, as no compounds have as yet been unambiguously identified that electrically connect cellular electron transport reactions to solid materials, such as electrodes. In eukaryotic microorganisms, electron transfer reactions are mainly related to the energy gain from the oxidation of reduced nicotinamide-adenine-dinucleotide (NADH) in the respiratory chain, which is localized in the mitochondria. The respective electron transfer compounds are embedded in the mitochondrial membrane. Moreover, they are shielded from extracellular electron acceptors, such as electrodes, by the plasma membrane and the cell wall, so that the accomplishment of direct contacts with electrodes seems unlikely. Bacteria are able to transfer electrons directly to

electrodes (mediatorless) either via the presence of the electro-active compounds (*cytochromes* for example) at the outer-cell membrane [10, 27], or via production of conductive nano-wires (*Pili*) [11, 12, 28]. Moreover, they are reported to produce and secrete low molecular weight compounds that act as electron shuttles and additionally have quorum sensing properties [13, 15, 29].

Thus, we wondered whether fungi also secrete metabolites with electron transfer properties. As these compounds might be present in the electrolyte solution, yeast cells were removed by centrifugation, and the cell-free solution was tested for electrochemical activity. Cyclic voltammetry resulted in an oxidation peak of the same height and position as the peak of the corresponding cell suspension (Fig. 5) showing that the electrochemical signal was independent of physical contact between the cells and the electrode.

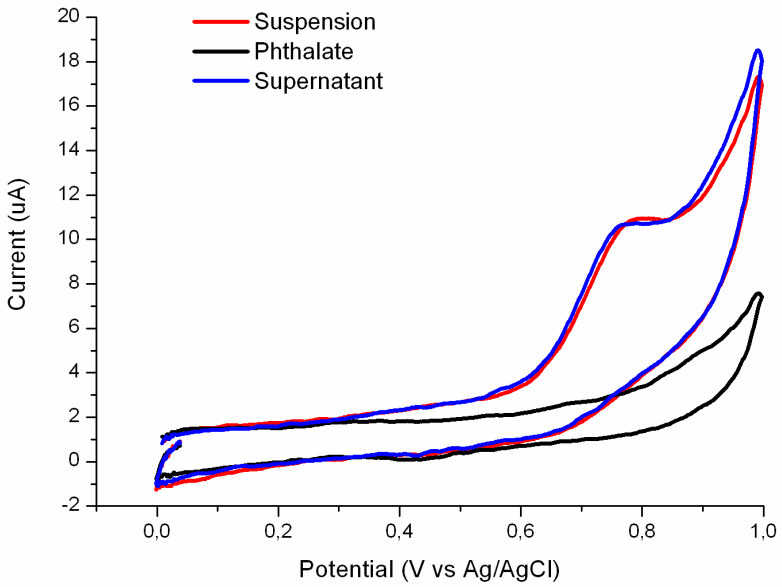

Figure 5: *C. albicans* cell suspension in phthalate buffer (electrolyte) in comparison with the blank phthalate buffer and the electrolyte solution, from which the cells were removed by centrifugation after their presence in the electrolyte for approximately 5 min (conditioned electrolyte). *C. albicans* was cultivated in YPD, the culture supernatant was removed by centrifugation, and the remaining pellet was resuspended in 1 ml PBS, which was added to the phthalate electrolyte.

3.2.2. Electrochemical behaviors of yeast quorum-sensing compounds

As in bacteria some quorum-sensing molecules function as electron shuttles, the electrochemical behavior of yeast quorum sensing compounds was tested. *C. albicans* produces various aromatic alcohols, which have quorum sensing properties or are involved in biofilm formation. Therefore, voltammograms of the alcohols farnesol [30], tyrosol [31, 32], phenylethanol [33], and tryptophol [34, 35] were recorded (Fig. 6a). Only tyrosol and tryptophol were electrochemically oxidized, leading to peak potentials at 630 mV and 740 mV, respectively. CVs of tryptophol were identical to those of *C. albicans* and *S. cerevisiae* cell suspensions (Fig. 6b).

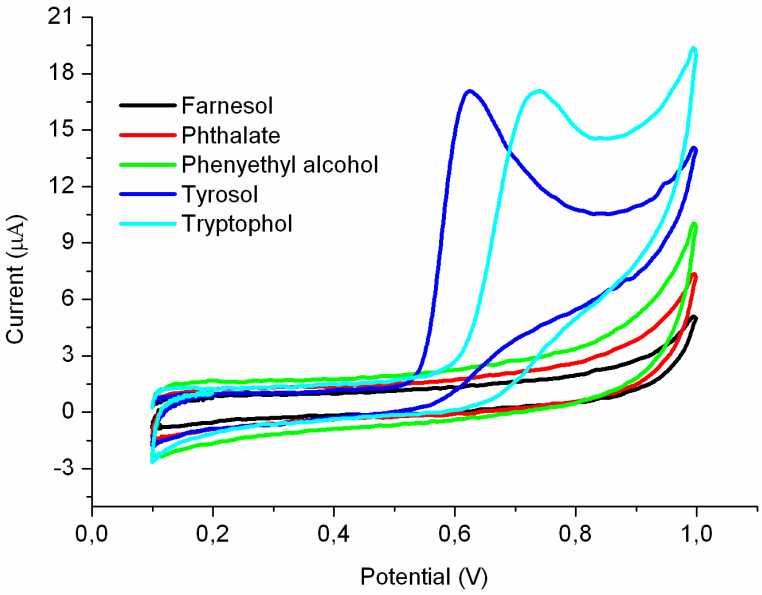

Figure 6a: Electrochemical behavior of the quorum sensing compounds, farnesol (7 µg/mL), phenylethyl alcohol (5 µg/mL), tyrosol (7.4 µg/mL), and tryptophol (4.3 µg/mL) in phthalate buffer pH 7; scan rate 50 mVs^{-1}.

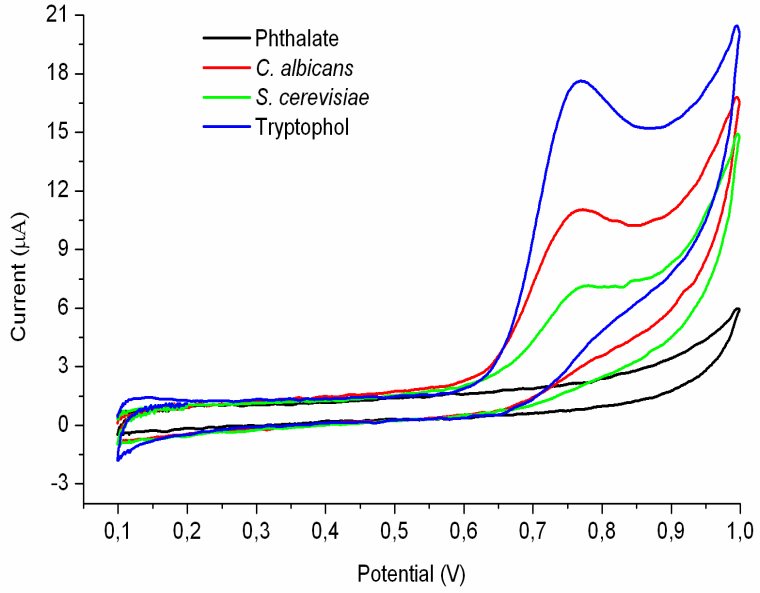

Figure 6b: The electrochemical behavior of tryptophol (4.3µg/l) in comparison with suspensions of *S. cerevisiae* and *C. albicans*. Blank phthalate buffer is shown as reference.

3.2.3. Tryptophol production increases the electrical signal

Tryptophol is a catabolic product of tryptophan [34], and the amount secreted by *C. albicans* into the electrolyte solution should depend on the availability of tryptophan. Thus, *C. albicans* was grown in amino acid free YNB medium supplemented with various concentrations of tryptophan. As a result, no oxidation peak was obtained when tryptophan-free YNB medium was used, whereas the oxidation peak was recovered where tryptophan was added to the YNB medium. The peak current strongly correlated with the tryptophan concentration (Fig. 7a). YPD medium is a complex medium, and the higher electrochemical signals resulting from *C. albicans* cultivated in this medium (Fig. 7b) can be explained by the sufficiently high amounts of all amino acids, including tryptophan.

LC/MS analysis (performed by H. Steinmetz, MWIS, HZI) of the conditioned electrolyte confirmed the presence of tryptophol in the electrolyte. Whereas no tryptophol could be detected when the cells were grown in YNB medium without tryptophan, increasing tryptophol concentrations were found when cells were grown in medium with increasing tryptophan concentrations (data not shown).

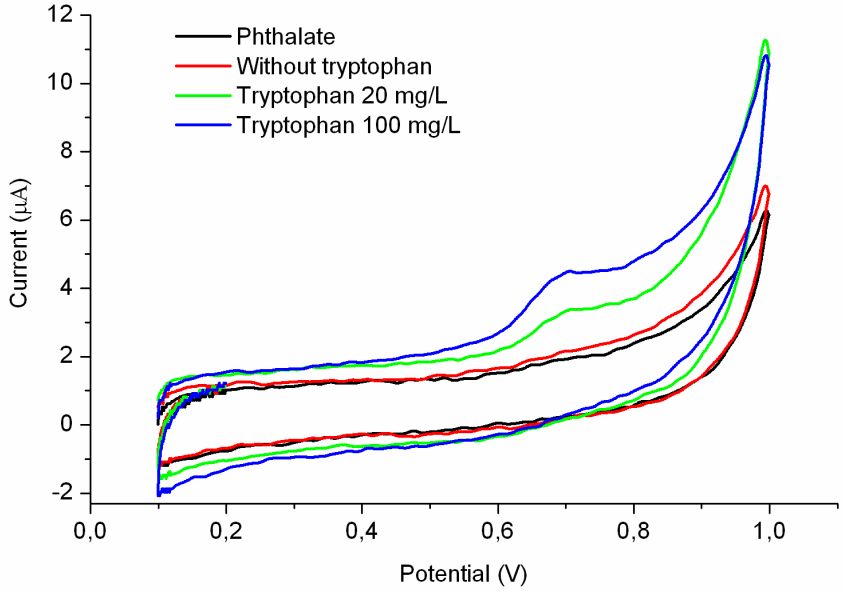

Figure 7a: Influence of tryptophan concentrations in YNB medium on the electrochemical signal of *C. albicans* suspension.

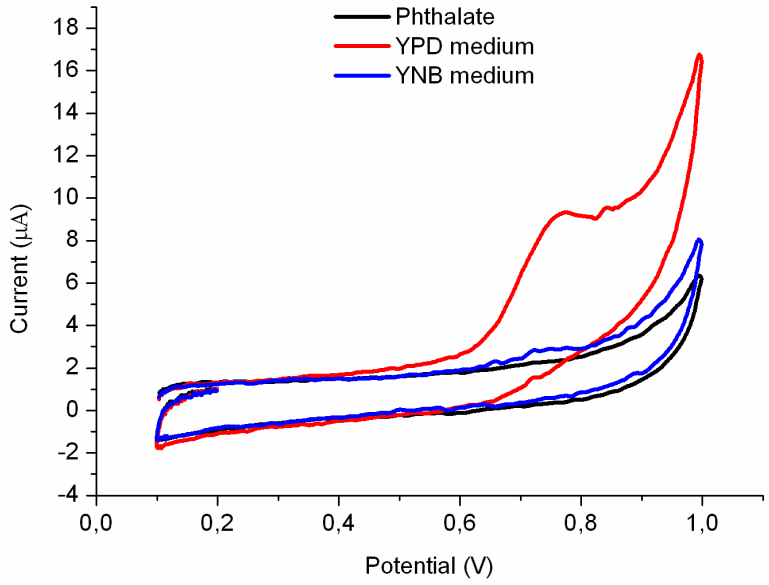

Figure 7b: CVs of *C. albicans* suspension grown in YPD medium and in YNB medium without amino acids, respectively.

3.3 Effects of the respiratory chain activity on the electrochemical signal

We established tryptophol as the source of the anodic oxidation currents of the yeast suspensions. As electron shuttling from yeasts to solid surfaces by secreted soluble compounds has not yet been reported, we studied the relationship of tryptophol concentrations to fungal electron transfer reactions, i.e., to the respiratory chain. We used the anodic peak current obtained from the yeast suspensions in the phthalate buffer as an indicator of tryptophol concentrations.

3.3.1. Activation of the respiratory chain

Electron transfer processes are related to the metabolic activity of the yeasts. As the electrolyte solution did not contain carbon sources, the metabolic activity of the suspended yeasts relied on internal stores. Thus, for the metabolic activation of the suspended cells, we supplemented the electrolyte buffer not only with the fermentable carbon source glucose, but also with the non-fermentable sugar galactose. For *S. cerevisiae*, the addition of glucose had almost no effect, whereas the current significantly increased in the presence of galactose (Fig. 8). Thus, the activation of metabolism was not sufficient to increase currents, but the activation of the respiratory pathways favored high oxidative currents.

For *C. albicans*, the oxidative peak current was independent of the carbon source added to the electrolyte solution, but the addition of the carbohydrates significantly increased the current in comparison with the plain buffer solution (Fig. 8).

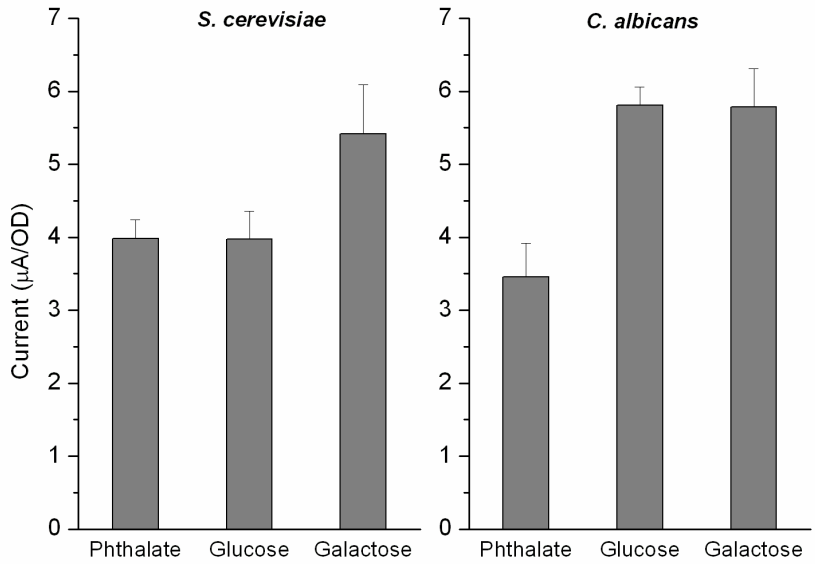

Figure 8: Influence of the metabolic activity of cells. Glucose or galactose was added to the phthalate buffer for the metabolic activation of the yeasts. Galactose was used as a non-fermentable carbon source, whereas glucose can also be consumed by fermentative pathways in *S. cerevisiae.*

3.3.2 Influence of electron transport chain inhibitors (ETC)

The most common inhibitors of complexes of the ETC are rotenone, antimycin A (AA), and cyanide. Rotenone inhibits the activity of complex I, whereas antimycin A interferes with the electron flow in the complex III. Cyanide generally affects all metalloenzymes, but its toxicity derives from its binding to Fe^{3+} in the heme groups of complex IV. We additionally used TTFA, which is a complex II inhibitor, and SHA an inhibitor of the AOX pathway. The effects of these inhibitors on the peak currents of both *S. cerevisiae* and *C. albicans* are shown in Fig. 9. As *S. cerevisiae* lacks complex I and AOX, rotenone and SHA were not applied to this yeast. Signals from the suspensions of both yeasts decreased when any of the major complexes of the classical respiratory chain, i.e., complex I, complex III, or complex IV, was inhibited. Inhibition of complex II had no effect on *S. cerevisiae* but reduced the signals from the *C. albicans* suspension. However, an increase in oxidation current of

C. albicans was observed after treatment with SHA (alternative respiratory inhibitor), as shown in Fig. 9a.

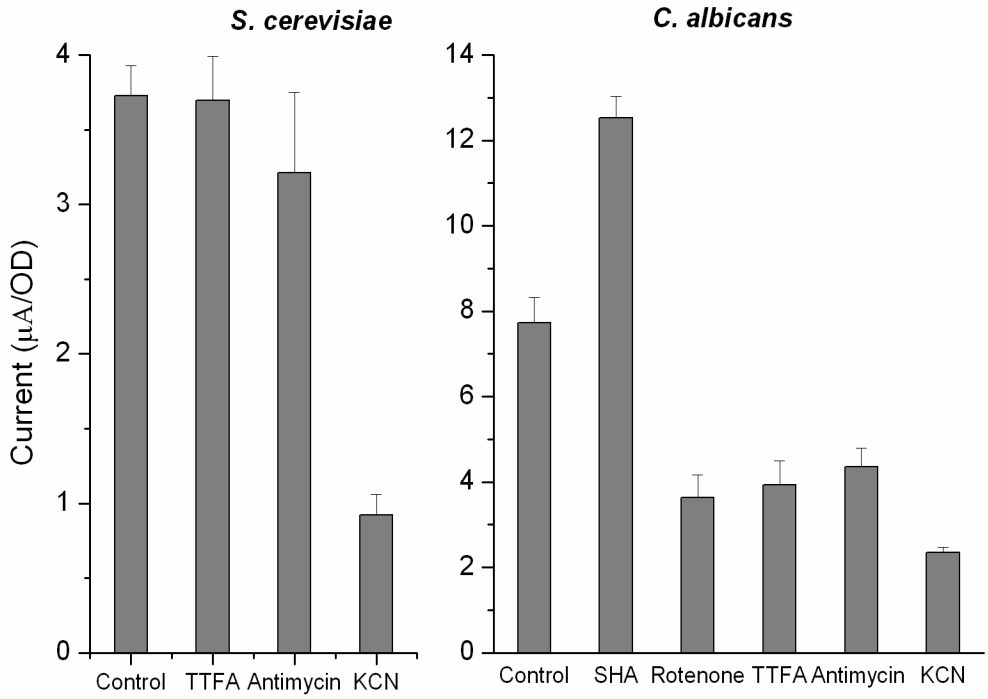

Figure 9: Effects of electron transport chain (ETC) inhibitors on the anodic peak currents of *S. cerevisiae* and *C. albicans.* Inhibitor concentrations were chosen on the basis of inhibitory effects on oxygen consumption: rotenone (41 µg/ml), thenoyltrifluoroacetone (TTFA) (5 µg/ml), antimycin A (1.5 µg/ml), KCN (5 µg/ml), and salicylhydroxamic acid (SHA) (14 µg/ml).

3.3.3 Activation of the alternative pathway (AOX)

C. albicans possesses two genes for AOX, of which one (*AOX2*) is differentially regulated in particular in response to the presence of ETC inhibitors [36]. Thus, we investigated the activation of AOX in our conditions by reverse transcription with polymerase chain reaction (RT-PCR) analysis of the expression of the *AOX1* and *AOX2* genes in *C. albicans*. *AOX1* expression was constitutive, and we proved the induction of *AOX2* by KCN, antimycin A, TTFA, and rotenone. SHA had no effect on *AOX2* expression (Chapter 2). Thus, the application of inhibitors of any of the complexes I – IV blocked the electron transfer through the classical respiratory chain, but allowed NADH oxidation via the AOX branch. SHA did

not lead to an additional capacity of this bypass of the classical respiratory pathway but inhibited its basal activity resulting from AOX1; hence, it led to increased electron flow through the classical respiratory chain.

These data show that the undisturbed performance of the classical respiratory chain, including complex IV (the terminal cytochrome c oxidase; COX), is essential for maximum signals, whereas the AOX pathway is not involved. Inhibition of the branched pathway via SHA even directs the residual electron flow to the classical pathway, thus enhancing the electrical signal.

Table 2: Inhibitors of the various components of the mitochondrial electron transport chain (mETC-inhibitors). The concentrations used for the treatment of *C. albicans* cultures resulted from investigations of inhibitory effects on oxygen consumption and the ROS production (data are shown in chapter 2). The peak currents from linear sweep voltammograms and cycle numbers from AOX2 gene expression analysis by RT-PCR were normalized with respect to data from cultures without inhibitors. (*$p<0.05$)

	Inhibitor	**Concentration (µg/ml)**	**Peak current vs control (%)**	**log_2 fold-change of *AOX2* expression compared with solvent control**
Complex I	Rotenone	41	70	3.5*
Complex II	Thenoyltrifluoroacetone (TTFA)	5	76	6.2*
Complex III	Antimycin A (AA)	1.5	84	7.0*
Complex IV	Cyanide (KCN)	5	45	11.0*
AOX	Salicylhydroxamic acid (SHA)	14	192	1.5

3.3.4 Effects of deletions of nuclear genes of COX subunits of S. cerevisiae

The application of the chemical inhibitors of the respiratory chain showed that the undisturbed function of the whole classical respiratory chain was essential for maximum tryptophol production. As a complementary approach, we investigated the effects of single gene deletions, focusing on the final complex of the electron transport chain, viz., cytochrome c oxidase (COX) because we had observed the most significant decrease of the peak current with the COX inhibitors KCN. This protein takes part in the transfer of electrons from cytochrome c *via* several steps to the terminal binuclear site involving heme a_3 and Cu_B (heme a_3–Cu_B), where catalysis of the reduction of O_2 to water takes place [37]. Subunits of COX, which are encoded by nuclear genes, contribute to the assembly of the whole enzyme and to the intra-molecular electron transfer rates [37]. Thus, we used *S. cerevisiae* single gene deletion mutants of the genes *COX5a*, *COX5b*, *COX7*, *COX8*, and *COX12* to elucidate the relevance of the different subunits for the electrochemical signal. As shown in Fig. 10a, the signal was reduced for all mutants. This correlated with the reduced respiratory activity as reported in literature for all these deletion mutants [38-40]. The strongest effect on the oxidation peak current was observed for the *COX5a* mutants, from which almost no signal could be detected.

To confirm the relevance of subunits *COX5a* and *COX5b* to the respiratory activity, oxygen consumption was determined for the respective mutants cultivated in YPgal medium (Fig. 10b). The respiratory activity was shown to be reduced if either of the two genes was deleted. This effect was more pronounced in the *COX5a* mutant. COX5a is described as an aerobic gene, whereas *COX5b* is a hypoxic gene, i.e., it is only expressed under low oxygen concentrations [41]. Thus, under our cultivation conditions, the expression level of the hypoxic gene *COX5b* probably was not high enough to fully compensate for the deletion of the aerobic gene *COX5a*, thereby leading to the strong effect on respiratory activity.

Overall, these data agree with the observations obtained with the chemical inhibitors, as the oxidative peak current increased with the increasing respiratory activity of yeast, and any interference by the deletion of a regulatory gene led to a reduction of signals.

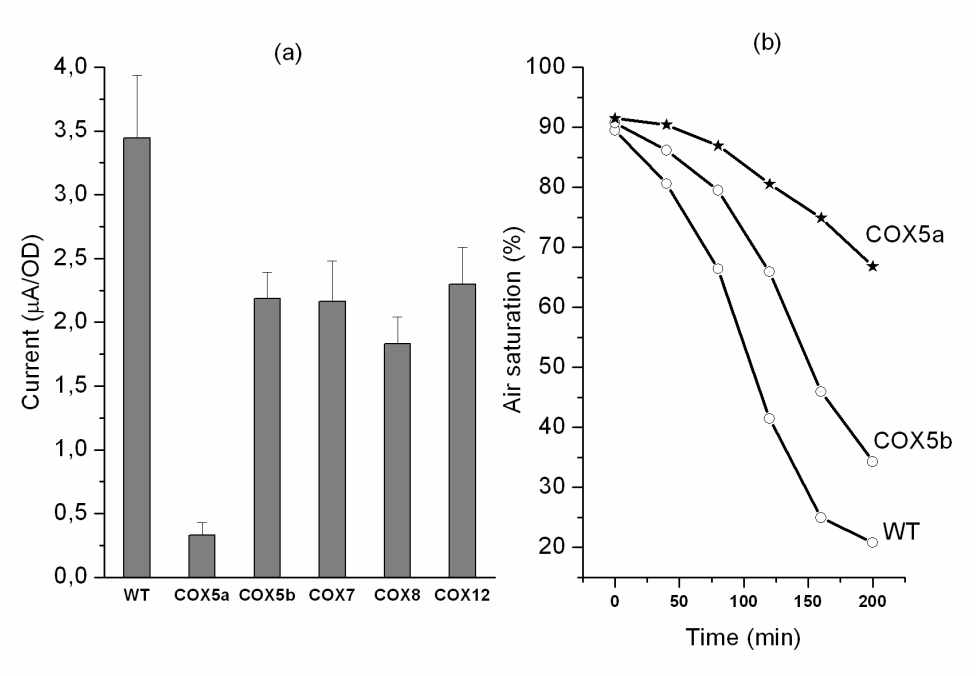

Figure 10: (a) anodic peak currents of *Saccharomyces cerevisiae* (WT) and single gene deletion mutants of cytochrome c oxidase COX subunits (cell numbers 2.3 x 10^6/ml, 2.3 x 10^6/ml, 2.3 x 10^6/ml, 2.71 x 10^6/ml, 2.6 x 10^6/ml, and 2.4 x 10^6/ml, for wild-type (WT) and *COX5a*, *COX5b*, *COX7*, *COX8*, and *COX12* mutants, respectively).

(b): Oxygen consumption of *S. cerevisiae* (WT) and of the *COX5a* and *COX5b* single gene deletion mutants in YPgal medium, and cell numbers of 5.6 x 10^6/ml, 5.9 x 10^6/ml, and 6.2 x 10^6/ml for *S. cerevisiae* (WT), *COX5a*, and *COX5b*, respectively.

4. Summary

The capability of microorganisms to transfer electrons to electrodes without the addition of exogenous mediators has been known for a long time. For bacteria, electron transfer has been shown to be achieved either by the production of soluble small-molecule electron transfer shuttles [13, 15] or by electron transfer proteins located in outer-membranes, so that direct physical contact to the electrode is possible. However, for yeasts, the respective mechanisms are largely unknown. As extracellular electron transfer compounds had not yet been identified, the existence of a trans-plasma membrane electron transport system [1] or the involvement of CoA from the cell wall [7] had been postulated. We have now shown, for the first time that the aromatic alcohol tryptophol acts as an electron shuttle for both *C. albicans* and *S. cerevisiae*. Tryptophol is one of the aromatic alcohols that are known as fusel oils in yeast and that result from catabolism of aromatic amino acids [35]. Their production is regulated by environmental conditions, such as oxygen concentration, pH, and the availability of nitrogen sources and the respective amino acids. The endogenous function of these secreted complex alcohols is not fully understood, but they seem to be involved in quorum sensing and biofilm formation [42]. Under yeasts growth conditions, only tryptophol is formed as a quorum sensing molecule. We have identified a new function of this alcohol, as it serves as an electron shuttle from the respiratory chain to the electrodes. Bacterial soluble electron transfer compounds, such as phenazines [13], are also involved in biofilm formation. They are assumed to allow microorganisms to obtain access to soluble reduced forms of essential metals, such as iron, which can be reduced from Fe^{3+}-oxides to Fe^{2+}-salts [43]. This capability is relevant mainly in biofilms or under other conditions of high cell density in which direct contact between iron sources and microbial cells might be difficult to achieve. As yeasts also form biofilms, the existence of a similar mechanism seems likely, and we have been able unambiguously to show that tryptophol is one of the relevant compounds.

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A viability assay for *Candida albicans* based on an exogenous electron transfer mediator system

Abstract

Rapid and specific diagnostic methods for detecting the viability of *Candida albicans*, an opportunistic fungal pathogen, are urgently needed. Electrochemical methods are considered to be sensitive methods for the viability determination of microorganisms. Thus, we established a viability test based on 2,6 dichlorophenolindophenol (DCIP), a membrane permeable electron transfer agent. NADH dehydrogenases catalyze the reduction of DCIP by NADH, and the enzymatic activity can be determined either electrochemically via oxidation reactions of DCIP or photometrically. Among the specific respiratory chain inhibitors only the complex I inhibitor rotenone decreased the DCIP-signal from *C. albicans* leaving a residual activity of approximately 30 %. Thus, the DCIP-reducing activity of *C. albicans* was largely dependent on complex I activity. *C. albicans* is closely related to the complex I-negative yeast *Saccharomyces cerevisiae*, which had previously been used in DCIP – viability assays. Via comparative studies, in which we included the pathogenic complex I-negative yeast *Candida glabrata*, we could define assay conditions, which allow a distinction of complex I-negative and -positive organisms. Basal levels of DCIP turnover by *S. cerevisiae* and *C. glabrata* were only 30 % of those obtained from *C. albicans*, but could be increased to the *C. albicans* level by adding glucose. No significant increases were observed with galactose. DCIP reduction rates from *C. albicans* were not further increased by any carbon source.

The proposed assay had been successful in the assessment of mitochondrial complex I activity in histidine kinase mutants of *C. albicans*. The bio-electrochemical behaviors of wild-type and *sln1* mutant revealed that Δ*sln1* mutant seems to have a defect in the function of the mitochondrial complex I (NADH-ubiquinone oxidoreductase).

1. Introduction

One of the most frequent human fungal pathogens is *Candida albicans*. It is the cause of oral and vaginal thrush as well as of severe mucosal and systemic infections in immunocompromised individuals [1]. Environmental safety concerns and needs in medical diagnosis to rapidly identify pathogenic organisms and their susceptibilities to antibiotics have prompted the development of a variety of new diagnostic methods [2, 3]. Molecular methods have the drawback that they do not distinguish between viable and non-viable organisms so that the infectious risk may be overestimated [2]. However, classical methods for the determination of viable microorganisms are based on the capacity of the organism to multiply and to form colonies, which has been questioned due to long analysis times, and the development of assays that rely on the metabolic activity rather than cell growth has been suggested [4]. Respiration [5] and electron transfer reactions [6-9] were considered suitable indicators of the metabolic activity of cells, as they are essential for proliferation of aerobic organisms.

In eukaryotic organisms the respiratory chain is located in mitochondria where more than 90% of the total oxygen is consumed via the electron transport chain (ETC). The classical respiratory chain (CRC) in eukaryotes comprises three large protein complexes: NADHubiquinone oxidoreductase (complex I), ubiquinol: cytochrome *c* oxidoreductase (complex III or cytochrome *bc1* complex) and cytochrome *c* oxidase (complex IV) (Fig. 1). In each complex electron transport is coupled to proton translocation, with the resultant proton motive force being used for ATP synthesis. Each complex can specifically be inhibited, e.g. complex I by rotenone, complex III by antimycin and myxothiazol and complex IV by cyanide. Electron transfer from succinate to ubiquinone without pumping of protons is done by succinate dehydrogenase (complex II) [10], of which thenoyltrifluoroacetone (TTFA) is a specific inhibitor. However, *C. albicans* has a more complex respiratory chain structure due to the presence of three respiratory pathways: In addition to the classical respiratory chain (CRC), an alternative oxidase [10-12] and a parallel electron transport chain (PAR) [13] were described, as shown in Fig. 1. The CRC comprises all four already described enzymatic complexes [10, 14], leading to coupling of proton translocation and NADH oxidation. The alternative respiratory pathway is generally conferred by a cyanide-insensitive alternative oxidase located on the matrix side of the inner mitochondrial membrane and encoded by two nuclear genes, *AOX1* and *AOX2*. *AOX1* is constitutively expressed, whereas expression of AOX2 is induced, for example by the presence of inhibitors of complexes of the CRC. These alternative oxidases catalyze the direct oxidation of ubiquinol by oxygen without proton

translocation and, thus, bypass complexes III and IV and enable respiration even in the presence of downstream CRC inhibitors. Due to the lack of a proton gradient energy storage from NADH oxidation is less efficient than in the CRC. AOX are inhibited by salicylhydroxamic acid (SHA) [12, 15]. The PAR is only activated when both the CRC and the AOX pathways are totally blocked, allowing electron flux to be redirected upstream of complex III, in parallel to the CRC [13].

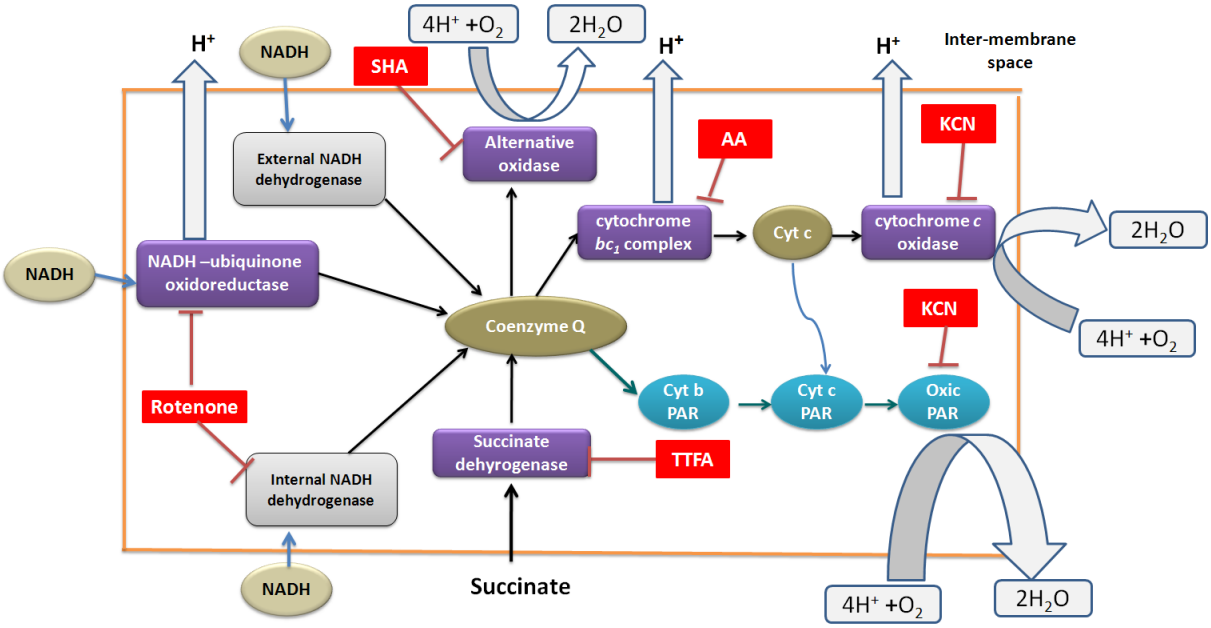


Figure 1: Scheme of the respiratory chain structure of *C. albicans.* NADH is oxidized by any of the NADH-dehydrogenases, in particular by the proton-pumping NADH-ubiquinone oxidoreductase (complex I), leading to reduced coenzyme Q. Coenzyme Q is also produced from the oxidation of succinate by succinate-dehydrogenase (complex II). Reduced coenzyme Q is oxidized either by oxygen through the alternative oxidase, or by cytochrome c in the cytochrome *bc1* complex (complex III). Cytochrome *c* is oxidized by oxygen by cytochrome *c* oxidase (complex IV). These latter reactions can also be catalyzed by components of the parallel pathway PAR. Inhibitors are known for each of the major enzyme complexes; SHA: salicylhydroxamic acid; AA: antimycin A; KCN: cyanide; TTFA: Thenoyltrifluoroacetone.

In other fungi, such as the pathogenic yeast *Candida glabrata* and the non-pathogenic yeast *Saccharomyces cerevisiae*, complex I is absent and so-called external and internal NADH dehydrogenases catalyze the oxidation of NADH without generation of a trans-membrane proton gradient. For simplicity we call these yeasts complex I– negative yeasts, to distinguish them from yeasts, which possess all four complexes of the CRC, such as *Candida albicans*,

which we call complex I – positive yeasts. Complex I – negative yeasts are usually rotenone-insensitive [16, 17]. One direct indicator of the respiratory activity of microorganisms is the oxygen consumption rate, and we had previously shown that *C. albicans* has a higher respiratory activity than *S. cerevisiae* [5]. Alternatively the activity of electron transfer reactions can be evaluated by artificial electron acceptors, and tetrazolium salts [8, 9, 18, 19], resazurin (AlamarBlue) [7, 20] and various quinoid compounds [6, 21-23] have been used to detect the viability of microbial and mammalian cells via color changes, which are usually related to the reduction of these compounds. However, also electrochemical detection methods are applicable, when the reduced electron acceptor can be oxidized at electrodes. 2,6-dichlorophenolindophenol (DCIP) is one of the compounds allowing both spectrophotometric [21-23] and voltammetric [24] detection of cellular reactions. It is known as photometric pH and redox indicator [25]. The reduced form is colorless, whereas the oxidized form has a dark blue color in solutions with pH > 6 and is pink in solutions with lower pH [25]. The reduced form of DCIP ($DCIPH_2$) contains two oxidizable groups, namely the 2,6-dichloro-4-quinone imino group and the phenolic ring. The oxidation of the quinone imino group is considered to be a reversible two-electron + two-proton transfer reaction and usually proceeds at potentials below 0.1 V (vs. Ag/AgCl reference electrode) [26]. In acidic solutions the formal potential is shifted to more positive values [25], and at a glassy carbon electrode even a clear separation of the anodic and the cathodic peak was observed [26]. Oxidation of the phenolic ring occurs at potentials higher than 0.5 V and is an irreversible reaction [26]. It is assumed that potentials higher than 0.4 V lead to electro-polymerization of DCIP with preservation of the redox properties for NADH [26, 27]. Most electrochemical assays with DCIP focus on the low potential range and use DCIP for mediated NAD(P)H detection [26, 27]. DCIP easily penetrates cell membranes and reacts with the intracellular NAD(P)H pool. This reaction is catalyzed by NAD(P)H dehydrogenases and DCIP is used as an indicator of NADH dehydrogenase activities, in particular of the activity of complex I [22-24]. However, DCIP was also used in a toxicity assay based on the complex I – negative yeast S. *cerevisiae*, as the absorbance of DCIP at 600 nm decreased with increasing metabolic activity of *S. cerevisiae* [22]. Thus there are also NADH-DCIP reductase activities, which are independent of complex I. We established a viability assay for *C. albicans* based on the electron transfer capabilities of DCIP and the electrochemical analysis of DCIP in the extended potential range from –0.25 V to +1.0 V. We were interested in an assay, which supported the analysis of structures of electron transport chains and of the mode of action of respiratory chain inhibitors in yeasts. Thus, we performed comparative studies between *C. albicans* and the complex I – negative

yeasts *C. glabrata* and *S. cerevisiae*. This enabled us to define assay conditions, which allow a distinction of complex I - positive and –negative yeasts. Moreover, the relevance of metabolic activation could be studied.

2. Materials and methods

2.1. Chemicals and reagents

Synthetic carbon powder and 2,6-dichlorophenolindophenol (DCIP) sodium salt were obtained from Sigma-Aldrich. A stock solution of 0.1 M of DCIP was prepared by dissolving the appropriate amount of DCIP in MQ water. The final concentration in the electrochemical cell was 40µM. YPD medium, antimycin A from *Streptomyces sp*., and rotenone were purchased from Sigma, and paraffin oil and 2-thenoyltrifluroacetone (TTFA) were obtained from Fluka. NADH and KCN were obtained from Roth (Karlsruhe; Germany) and Merck (Darmstadt, Germany), respectively. All chemicals were of analytical grade and used without further purification.

2.2. Microorganisms and growth conditions

The following microbial strains were used: *C. albicans* CAF2-1 [28], which was derived from *Candida albicans* SC5314 (ATCC MYA-2876), and *S. cerevisiae* BY4741. Yeast cells were cultivated overnight in 250 ml flasks in 50 ml YPD medium (yeast extract (10 g/l), peptone (20 g/l), and glucose (20 g/l)) at 30° C. A pre-culture was prepared by diluting the overnight culture to an optical density (OD_{620}) of 0.2 in 25 ml YPD and the yeast cells were allowed to grow for 3 hours so that they reached the exponential growth phase. The OD was determined in 180 µl sample volume with the microtiter plate reader µQuant (BioTek Instruments GmbH, Bad Friedrichshall, Germany). The working culture was prepared by diluting the pre-culture to an OD_{620} of 0.2. After cultivation for another 3 hours, the OD_{620} was recorded and the whole suspension was taken into a falcon tube and cells were harvested by centrifugation (Eppendorf centrifuge 5804R) at room temperature at 5000 rpm for 5 min, and washed carefully three times with PBS. The washed cells were resuspended in 1 ml PBS. To test the electrochemical activity of non-living organisms (dead cells), the working culture was autoclaved at 121°C for 20 min. The cells were harvested, washed and resuspended by the same procedure as described above.

2.3. Voltammetric procedure and preparation of carbon paste electrode

All electrochemical measurements were performed using a computer controlled Gamry Potentiostat/Galvanostat/ZRA G750, which was connected to a three electrode system comprising a carbon paste working electrode, a Pt disc auxiliary electrode and an Ag/AgCl/3M KCl reference electrode. The carbon paste electrode was prepared by thoroughly mixing 1 g of synthetic carbon powder 1-2 micron with 0.4 ml paraffin oil in a

small hand mortar. The hollow electrode (5mm) was filled with the carbon paste. For regeneration and cleaning a small part of the paste was cut off and the electrode surface was polished with a wetted filter paper. The working electrode was electrochemically activated prior to measurements by applying ten cyclic scans from 0.0 to 1.0 V with a sweep rate of 50mV/s in PBS buffer (pH 7) as a supporting electrolyte. Then 1 ml of the washed yeast cells were introduced into the electrochemical cell containing 24 ml PBS buffer with 40µM DCIP. All electrochemical experiments were carried out at room temperature. The incubation time of the cell suspension with DCIP usually was 5 minutes before linear sweep voltammograms (LSV) or cyclic voltammograms (CV) were obtained. Anodic peak currents were determined as absolute values, i.e. they were not related to a baseline resulting from other oxidation reactions occurring at lower potentials.

2.4. Spectrophotometric assay of the viability of yeasts

For validation of the electrochemical data, also spectrophotometric DCIP assays with the chosen yeasts (*C. albicans*, *C. glabrata* and *S. cerevisiae)* were performed. In each well of a 96-well plate (BD Falcon, New Jersey, USA) 180 µL of the cell suspension with OD_{620} of 0.5 was mixed with DCIP (in PBS) to result in a final concentration of 40 µM. The plate was incubated on a plate shaker at 30 °C for 20 min and the DCIP color at 600nm was determined using the microtiter plate reader µQuant (Biotek Instruments). The color change was calculated according to equation 1:

$$\textbf{DCIP color change (\%)} = 100 - (A600_{(20\ min)} / A600_{(0\ min)}) * 100) \qquad \text{(equation1)}$$

2.5. Oxygen determination of yeasts

The consumption of oxygen was monitored during the cultivation of yeast cells in YPD medium according to a previously described procedure [5]. Oxygen was determined with round-bottomed OxoPlates (PreSens, Regensburg, Germany) following the procedure given by the manufacturer. Briefly, the fluorescence of the indicator and the reference dye were determined from the bottom of the plates at excitation wavelengths λex of 530 nm and emission wavelengths λem of 620 nm for the indicator dye and λex 530 nm and λem 590 nm for the reference dye. Calibration was performed with water saturated with air (100%) and with an aqueous Na_2SO_3 solution (10 g/l) (0%) according to the supplier's protocol.

2.6. Effects of electron transfer chain inhibitors

C. albicans cells were treated with electron transport chain (ETC) inhibitors using the final concentrations 41 µg/ml rotenone, 5 µg/ml 2-thenoyltrifluoroacetone (TTFA), 14 µg/ml salicylhydroxamic acid (SHA), 1.5 µg/ml antimycin A (AA) and 5 µg/ml cyanide (KCN). The concentrations were chosen for each compound on the basis of the inhibitory effects on oxygen consumption and reactive oxygen species (ROS) induction. These inhibitors were added to the medium of the working culture (YPD). The yeast cells were allowed to grow in the presence of the inhibitors at 30 °C for 3 h and were harvested and resuspended as described above.

2.7. Metabolic activation

The metabolism of yeast cells (*C. albicans*, *C. glabrata* and *S. cerevisiae*) was activated by the addition of glucose or galactose to the PBS buffer. 2% glucose or galactose solutions were prepared in PBS. Sterilization was done by autoclavation. 1 ml of the washed yeast cells was transferred into a flask with 24 ml of the carbohydrate buffered solution and left on the shaker for 30 min at 30 ^{o}C. Then the electron transfer activities of the organisms to DCIP were electrochemically and photometrically determined by the addition of DCIP and incubation for 5 min and 20 min, respectively. Signals from cultures without carbohydrate supplement were used as control.

The rotenone-insensitive DCIP - reducing activity of the yeasts in the presence or absence of different C-sources was determined photometrically in 96 well plates. Each well of a 96-well plate was filled with 140 µl of PBS supplemented with or without C-source and with or without rotenone (41 µg/ml) or the appropriate volume of solvent (DMSO). 20 µl of the cell suspension were added to each well, and the plate was incubated at 30 ^{o}C on the plate shaker for 30 min. Finally, 20 µL of DCIP were added to a final concentration of 40 µM. The change of the blue color was monitored at 600 nm after 20 min.

2.8. Determination of NADH

S. cerevisiae and *C. albicans* were grown in YPD at 30 ^{o}C for 3 h and the corresponding OD620 was measured (working culture). The cells were collected by centrifugation, washed and re-suspended in PBS as described before. The supernatant was transferred into a new falcon tube. NADH was quantified in both the cell suspension and the supernatant. 5 µl of the cell suspension or the supernatant were placed in a 96 well plate (Costar, New York, USA) and 45 µl of PBS were added to obtain a final volume to 50 µl. The fluorescence intensity was quantified by a plate reader (Synergy 4, BioTek Instruments GmbH) at the excitation wavelength λex 370 nm and the emission wavelength λem 460 nm. An NADH calibration curve was obtained from a dilution series of NADH standard solutions.

2.9. Determination of reactive oxygen species (ROS)

The amounts of reactive oxygen species (ROS) produced in the yeast cells were measured by a fluorometric assay using H_2DCFDA,SE (2',7'-dichlorodihydrofluorescein diacetate, succinimidyl ester) (Invitrogen) as a Reactive Oxygen Species (ROS) indicator as previously described [29]. The working culture was prepared by diluting the pre-culture to an OD_{620} of 0.2 in fresh YPD medium. After cultivation for another 3 hours, the OD was measured; cells were harvested by centrifugation for 5 min at 5000 rpm at room temperature, and carefully washed with MQ water three times. Washed cells were resuspended in sterile water to an OD_{620} of 0.5. H_2DCFDA, SE was added from a stock solution of 2 mg/ml in DMSO to a final concentration of 40 µg/ml. After 30 min of incubation at 30 °C, the stained cells were collected by centrifugation and re-suspended again in sterilized MQ water. 120 µl of the stained cells were taken into a 96 well plate containing 60 µl of rotenone (final concentration was 41 µg/ml). The fluorescence intensity was quantified after 2 h using a microtiter plate reader (Synergy 4, BioTek Instruments GmbH) at the excitation wavelength λex 485 nm and the emission wavelength λem 535 nm.

3. Results and discussion

3.1. Electrochemical reaction of DCIP with yeasts

The cyclic voltammogram of DCIP showed two clearly defined oxidation peaks at +0.1 V and +0.5 V, and a reduction peak at +0.05 V, indicating the reversible nature of the first oxidation step (Fig. 2a) [26]. The addition of *C. albicans* led to a spontaneous increase of both oxidation peak currents. Additionally, the first peak became broader, and the second peak shifted by almost 0.1 V to +0.6 V (Fig. 2b). With the extension of the reaction time of *C. albicans* with DCIP (electrode switched off) to 15 min the first peak became less sharp and developed to a wave, of which the steady state current was almost identical to the original peak current (Fig. 2). In contrast, the peak form corresponding to the second oxidation step did not change and the peak current increased with reaction time at least comparable to the increase of the steady state level of the first oxidation step. These changes were also observed, when NADH was incubated with DCIP (Fig. 2c). After addition of DCIP the above mentioned anodic and cathodic peaks from DCIP oxidation (+0.1 V; +0.5 V) and reduction (+0.05 V) appeared in addition to the peak from NADH oxidation (+0.4 V). However, after a reaction time of 5 min the NADH peak was disappeared and the second DCIP oxidation peak was shifted to +0.6 V.

As the second peak remained sharp and well defined in all experiments and the peak current also was an indicator of the reaction of the yeasts with DCIP, in following investigations also linear sweep voltammetry in the potential range from 0.2 V to 1.0 V was used. Fig. 3 shows the linear sweep voltammograms of *C. albicans* and *S. cerevisiae* with DCIP in the anodic potential range at different incubation times of the yeast cells with DCIP (0, 5, 10 and 15 min). As observed in cyclic voltammograms (Fig. 2b) an anodic peak was observed at 600 mV. The oxidation peak current increased by more than 50 % with increasing the incubation time of DCIP with *C. albicans* to 15 min (from approximately 16.5 µA to approximately 29 µA), whereas only a minor increase in the oxidation peak current of DCIP incubated with *S. cerevisiae* was observed (approximately 10 % in 15 min). Thus, *S. cerevisiae* cells were significantly less capable to reduce DCIP.

No electrochemical response was observed when dead cells of *C. albicans* were used. Therefore, the anodic peak current at +0.6 V was obviously an indicator for the reduction of DCIP by viable *C. albicans* cells.

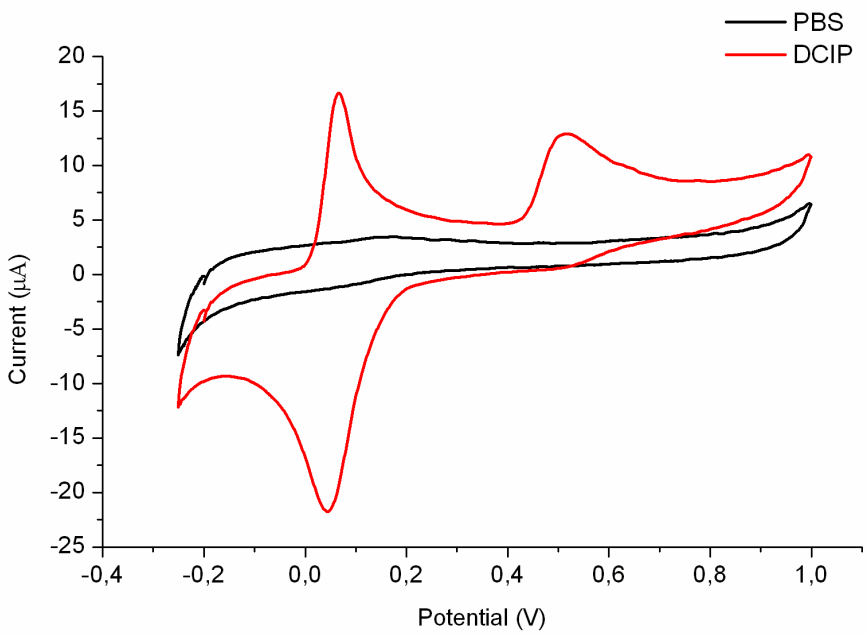


Figure 2a: Cyclic voltammogram of DCIP (40 µM) in PBS. The electrode was immersed in the DCIP solution for 5 min before the voltammogram was recorded.

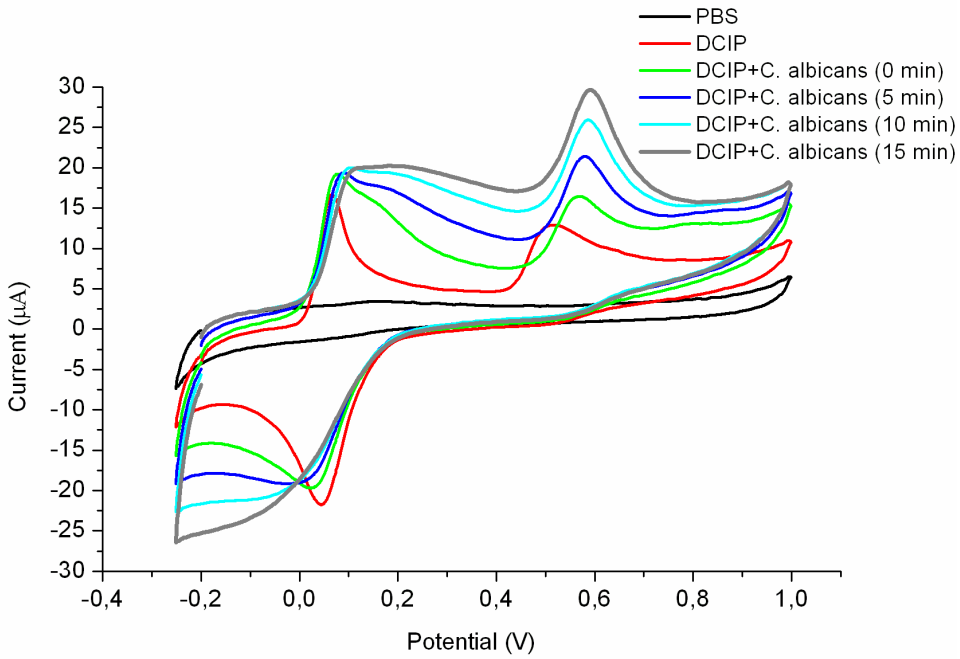


Figure 2b: Cyclic voltammogram of DCIP with *Candida albicans.* A cell concentration of 5 x 10^6 cells/ml was used. The voltammograms were recorded after the indicated incubation times, with the electrode being switched off during incubation.

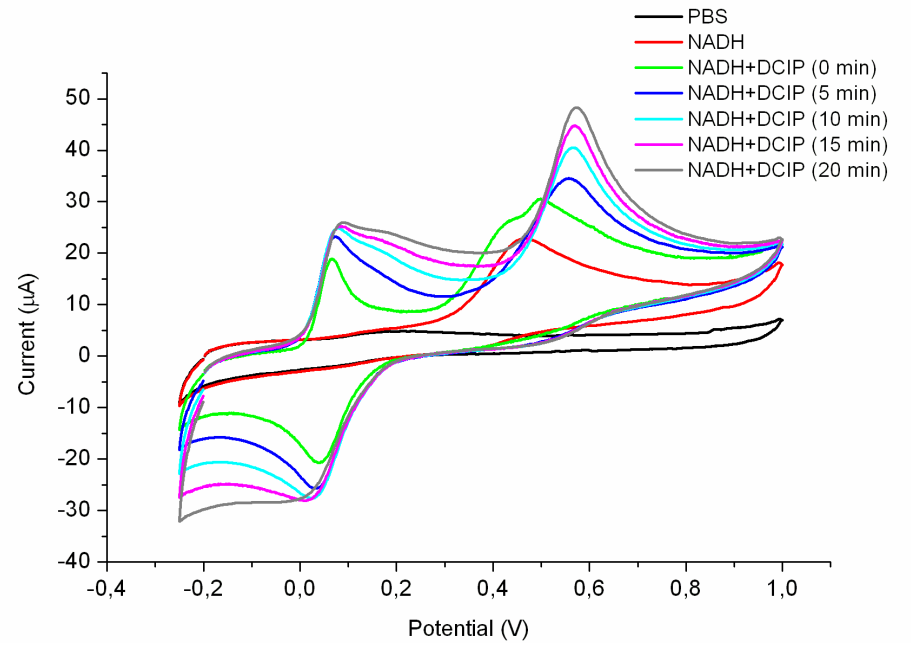


Figure 2c: Cyclic voltammogram of NADH (40 µM) incubated with DCIP in PBS. In all experiments the scan rate was 50 mV/s and representative voltammograms of repeated independent experiments are shown.

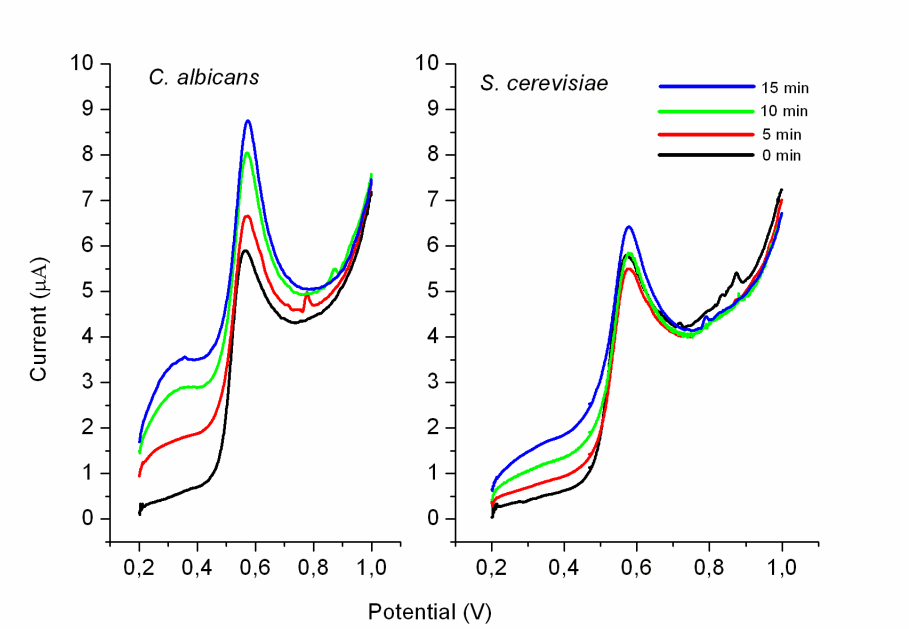


Figure 3: Linear sweep voltammograms of DCIP (40µM) incubated with *C. albicans* and *S. cerevisiae* after different incubation times (0, 5, 10, and 15 minutes) in PBS. The scan rate was 50 mV/s; the OD_{620} of each microbial suspension in the electrochemical cell was 0.5.

3.2. Peak current correlated with cell numbers and growth rate of C. albicans

The detection of reduced DCIP via the anodic peak current at 0.6 V provides a good bioelectrochemical system for monitoring the growth and viability of *C. albicans*. Figure 4 shows the dependence of the peak current on the number of viable cells. The current increased in the range from 2.6 x 10^6 to 4.5 x 10^7 cells/ml. Thus, a growth curve could be recorded during a cultivation period of 6h showing the three typical growth phases, i.e. the lag phase, the exponential or log phase and the stationary phase. In contrast to the estimation of cell numbers from OD_{620} – measurements, these electrochemical data indicate the increase of numbers of only viable cells.

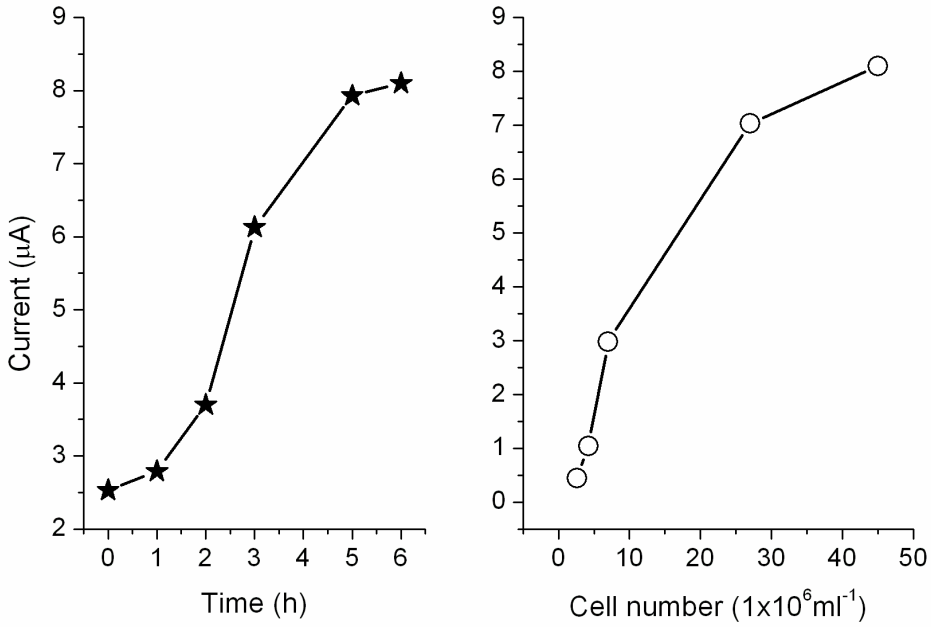


Figure 4: Increase of the peak currents due to electrochemical DCIP re-oxidation during growth of *C. albicans*. The relationship of cell numbers to the oxidation peak current of DCIP (40 µM) is also shown.

3.3. Assay specificity

DCIP is reduced by a number of different organisms, ranging from mammalian cells and yeasts to bacteria, which is usually observed via the decolorization of the blue dye. It is assumed that enzymes of the respiratory chain, and in particular NADH dehydrogenases, are involved in this reaction. Thus, we used the common inhibitors of the classical respiratory chain, namely rotenone, TTFA, antimycin A, and cyanide, and also SHA as inhibitor of the

alternative oxidase to identify the relevant electron transfer steps**.** Of these inhibitors only rotenone, the complex I-inhibitor, decreased the signals from reduced DCIP (figure 5). Residual anodic peak currents of approximately 30 % and residual absorbance changes of almost 50 % were observed (Table 1). Thus, in *C. albicans* the major complex involved in the reduction of DCIP was the rotenone-sensitive complex I, whereas the other complexes of the classical respiratory chain did not contribute. However, in previous reports DCIP was used as indicator of the metabolic activity of the complex I-negative yeast *S. cerevisiae* [22]. Thus, for comparison and further investigations on the specificity of the assay we included *S. cerevisiae* and the pathogenic complex I -negative yeast *C. glabrata* [17] in our studies. As previous toxicity assays were based on photometric detection, which could deliver different results by color changes due to pH changes, we established the photometric assay for *C. albicans* for comparison. Moreover, we investigated the respiratory activity of the yeasts by analysis of the oxygen consumption rates. Incubation of DCIP with yeast cells led to a decolorization of the blue color of DCIP. It was observed that the blue color of DCIP (oxidized form) was consumed very fast and turned into the colorless (reduced form) in the presence of *C. albicans*, however the rates of blue color change were very low when DCIP was incubated with *C. glabrata* or with *S. cerevisiae* (Fig. 6A). Similar ratios were obtained for the electrochemical signals from the different yeasts, as shown in Fig. 6B. Thus, we could show that indeed the presence of complex I is not an essential prerequisite for the reduction of DCIP, even though reaction rates were the highest in the complex I - positive yeast *C. albicans.* As we had observed a higher DCIP - reducing activity for *C. albicans* than for *C. glabrata* and *S. cerevisiae*, we analyzed the respiratory activities of the yeasts by measuring the oxygen uptake rates [5]. *C. albicans* consumed most of the dissolved oxygen during the first 20 min, whereas after the same incubation time still more than 60 % and 80 % dissolved oxygen was left when *C. glabrata* and *S. cerevisiae* were cultivated (Fig. 7), indicating a significantly higher respiratory activity of *C. albicans*. However, the cultivation medium YPD is a glucose rich medium, in which Crabtree–positive yeasts, such as *S. cerevisiae* and *C. glabrata*, may use fermentation pathways even in the presence of oxygen, so that oxygen consumption rates were reduced.

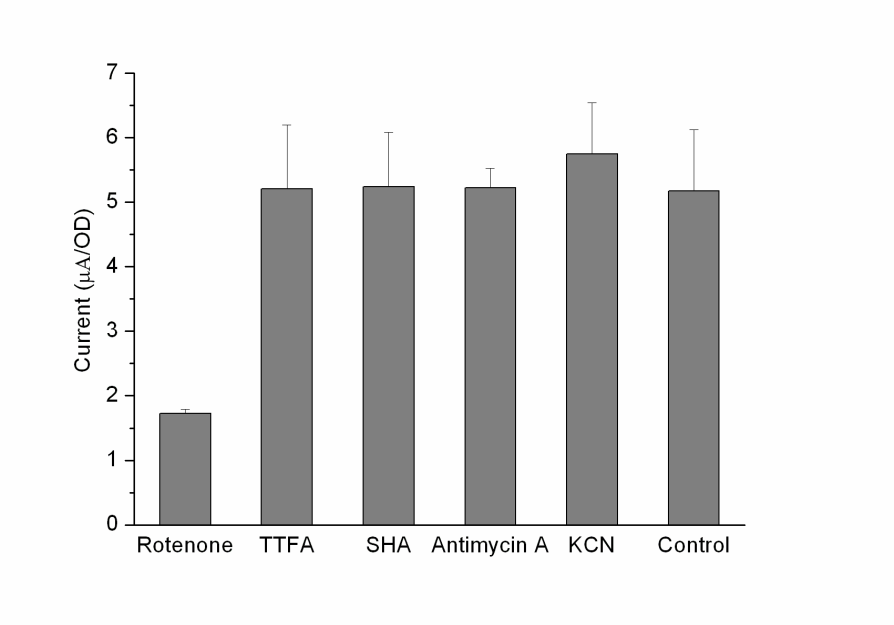


Figure 5: Effects of electron transport chain (ETC) inhibitors on the oxidation currents of *C. albicans.* Inhibitor concentrations: rotenone (41 µg/ml), thenoyltrifluoroacetone (TTFA) (5 µg/ml), antimycin A (1.5 µg/ml), KCN (5 µg/ml), and salicylhydroxamic acid (SHA) (14 µg/ml).

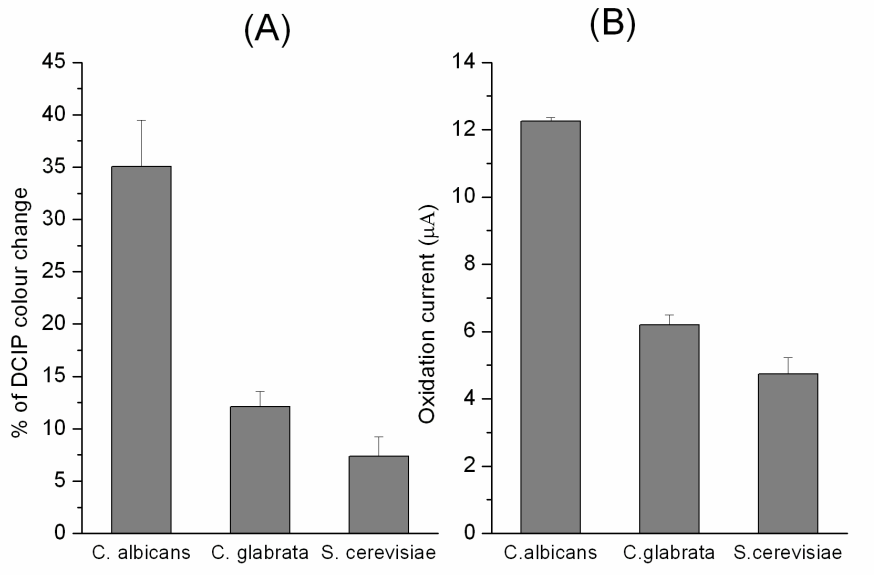


Figure 6: (A) Colorimetric assay of the DCIP-reducing activity of *C. albicans*, *C. glabrata* and *S. cerevisiae*, the incubation time of the yeasts with DCIP in the 96-well plate was 20 min. (**B)** Electrochemical detection of reduced DCIP after incubation with the different yeasts for 30 min.

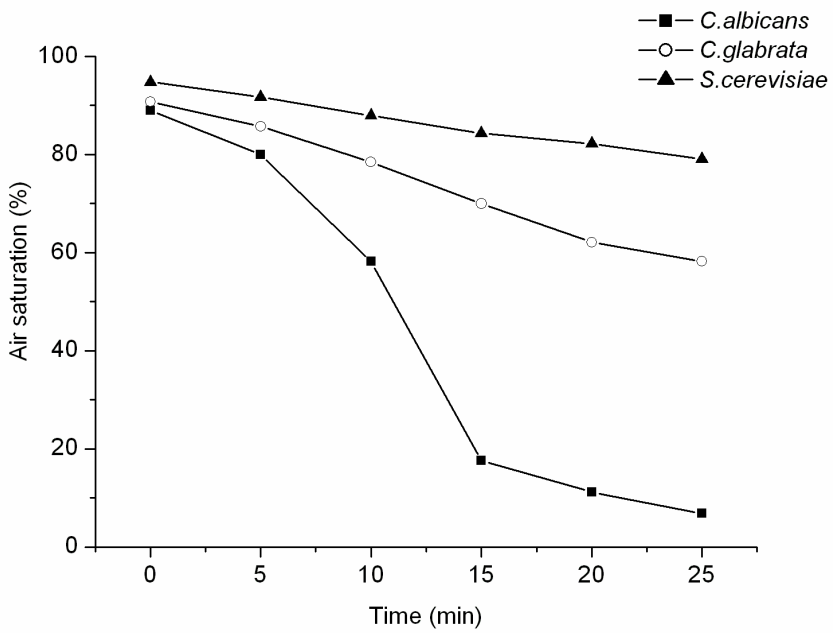


Figure 7: Oxygen consumption of *C. albicans*, *C. glabrata* and *S. cerevisiae* incubated in YPD medium. Oxygen concentrations are given as % air saturation. Starting OD_{620} of *C. albicans* and *C. glabrata* was 0.05. For *S. cerevisiae* an OD_{620} of 0.1 was used.

3.4. Metabolic activation of the yeasts

The incubation of the yeasts with DCIP was performed in an electrolyte solution, which did not contain carbon sources, so that the metabolic activity of the suspended yeasts relied on internal nutrient stores. In order to test the effects of metabolic activation by addition of different carbon sources on the DCIP reduction rate, we supplemented the incubation buffer with glucose or galactose. Data are summarized in Table 1. For *C. albicans*, the oxidative peak current was almost independent of the addition and types of carbon sources. Effects were slightly stronger, when DCIP reduction was followed photometrically, as the colour change rate of 22 % increased to 35 % after the addition of glucose. However, the signals from *S. cerevisiae* and *C. glabrata* increased to the 3- to 4-fold value in the presence of glucose, reaching almost the same level as the signals from *C. albicans* (Figure 8). The addition of galactose had no or minor effects on the DCIP re-oxidation peak currents from *S. cerevisiae* and *C. glabrata*. The activity of DCIP-NADH reductases, among which is complex I, in *C. albicans* obviously reached almost the maximum value independent of the additional stimulation of metabolism by carbon sources. However, only the presence of the fermentable carbon source glucose activated the DCIP-NADH reductase activity in the Crabtree-positive and complex I-negative yeasts *S. cerevisiae* and *C. glabrata*, whereas the non-fermentable

carbon source galactose had significantly smaller effects. Thus, in these yeasts DCIP reduction by the NADH pool was not strongly related to the activity of the respiratory chain, but may be due to the increased cellular NADH levels resulting from less efficient NADH oxidation during fermentation.

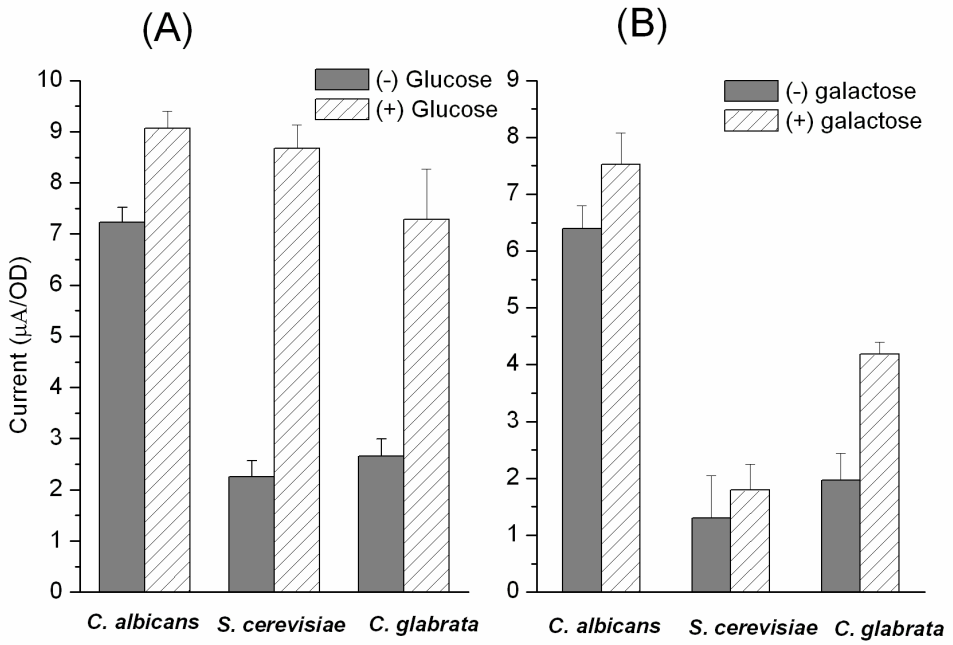


Figure 8: Effects of glucose (A) and galactose (B) on the electro-catalytic activity of *C. albicans*, *C. glabrata* and *S. cerevisiae*.

Table 1: Influence of the addition of glucose or galactose and of the complex I – inhibitor rotenone (41 µg/mL) on the DCIP reduction rates of the different yeasts; determined via the DCIP - oxidation currents (electrochemistry, after 5 min) or via absorbance changes (after 20 min). Values were normalized with respect to the OD_{600} of the cell suspension to consider different cell densities.

Yeast	Without carbon sources				Glucose			Galactose [µA]
	Electrochemistry		Absorbance			Absorbance		
	Control [µA]	(+) Rotenone [µA]	Control [%]	(+) Rotenone [%]	Control [µA]	Control [%]	(+) Rotenone [%]	
C. albicans	7.2 ± 0.7	1.7 ± 0.1	22 ± 4	11 ± 3	9 ± 2	35 ± 5	23 ± 1	7.5 ± 0.5
C. glabrata	2.3 ± 0.6	--	11 ± 3	14 ± 1	7.3 ± 0.9	31 ± 5	32 ± 5	4.3 ± 0.3
S. cerevisiae	2.2 ± 0.7	--	9 ± 3	9 ± 2	8.7 ± 0.8	32 ± 4	35 ± 5	1.7 ± 0.3

3.5. Effect of rotenone after metabolic activation

The DCIP-NADH reductase activity of *C. albicans* without metabolic activation could be inhibited by rotenone to approximately 30 %. Metabolic activation of *S. cerevisiae* and *C. glabrata* by glucose increased the DCIP reductive activity to a similar level as in *C. albicans*. Therefore, we investigated the effects of rotenone on the DCIP reducing activity of all yeasts after addition of glucose. We observed no inhibitory effect of rotenone on the DCIP reaction with *C. glabrata* and *S. cerevisiae*, irrespective of the presence of glucose, while the rotenone inhibitory effect was significant for *C. albicans* (Figure 9).

However, in the absence of glucose the rotenone-sensitive contribution to the overall color consumption rate was 70- 50 %, whereas in the presence of glucose it was only approximately 30 % (Tab. 1).

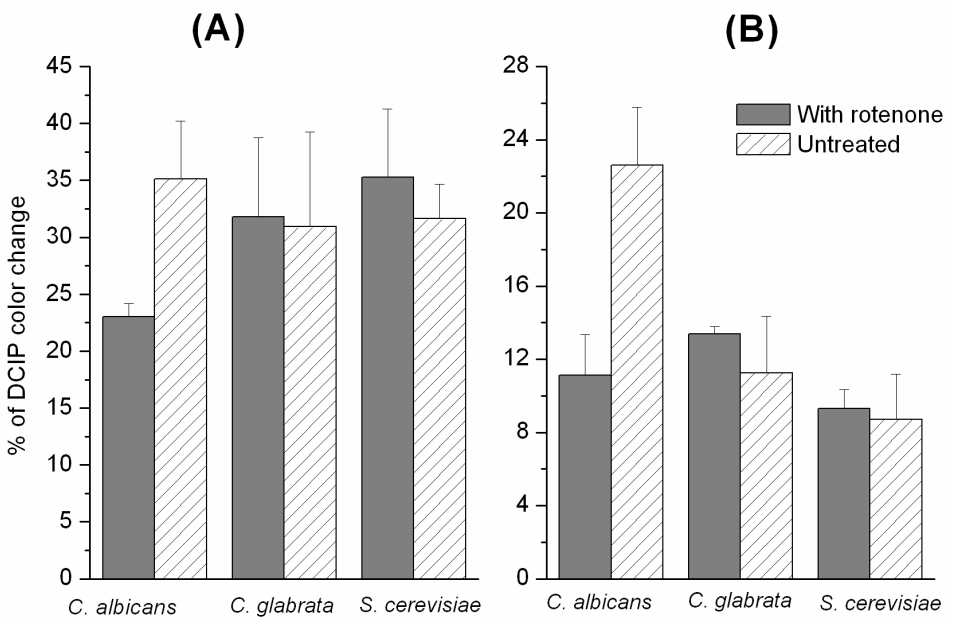


Figure 9: (A) Effects of rotenone on the DCIP reaction with yeasts, buffer supplemented with 2 % glucose. **(B)** Effects of rotenone on the DCIP reaction with yeasts in the absence of glucose.

3.6. Investigations of correlations to NADH concentrations in cells and supernatants

As the reducing agent of DCIP in the living cells is NADH, we investigated the correlation between NADH concentrations and the electrochemical signals. As already shown in Figure 2c, also a direct, non-catalyzed reduction of DCIP by oxidation of NADH is possible and we wondered whether the presence of cells was required or whether secreted NADH might be the source of the observed signals. We determined extra- and intra-cellular concentrations of NADH in *C. albicans* and *S. cerevisiae* via NADH fluorescence, and found a significantly higher NADH fluorescence in the supernatants, i.e. extracellular NADH-concentrations for both *S. cerevisiae* and *C. albicans* were higher in the supernatants than in the cell suspensions (Tab. 2). However, the consumption rates of the DCIP blue color (reduction of DCIP) were faster by the cell suspensions than by the supernatants, in particular by *C. albicans* (Tab. 2). Hence, it can be concluded, that the redox reaction of DCIP with living organisms is not only related to NADH availability, but is controlled by the enzymatic activity of NADH dehydrogenases.

Table 2: Relationship between NADH concentrations and DCIP reduction rates: NADH concentrations in the supernatants were higher than in the cells; however, the DCIP reduction rates determined from the yeast suspensions were higher than from the supernatants, showing the catalytic effect of the DCIP – NADH reductases.

Candida albicans				***Saccharomyces cerevisiae***			
Cells		Supernatant		Cells		Supernatant	
NADH (mM)	Color change (%)	NADH (mM)	Color change (%)	NADH (mM)	Color change (%)	NADH (mM)	Color change (%)
0.1	14	0.2	4	0.03	6.4	0.25	4

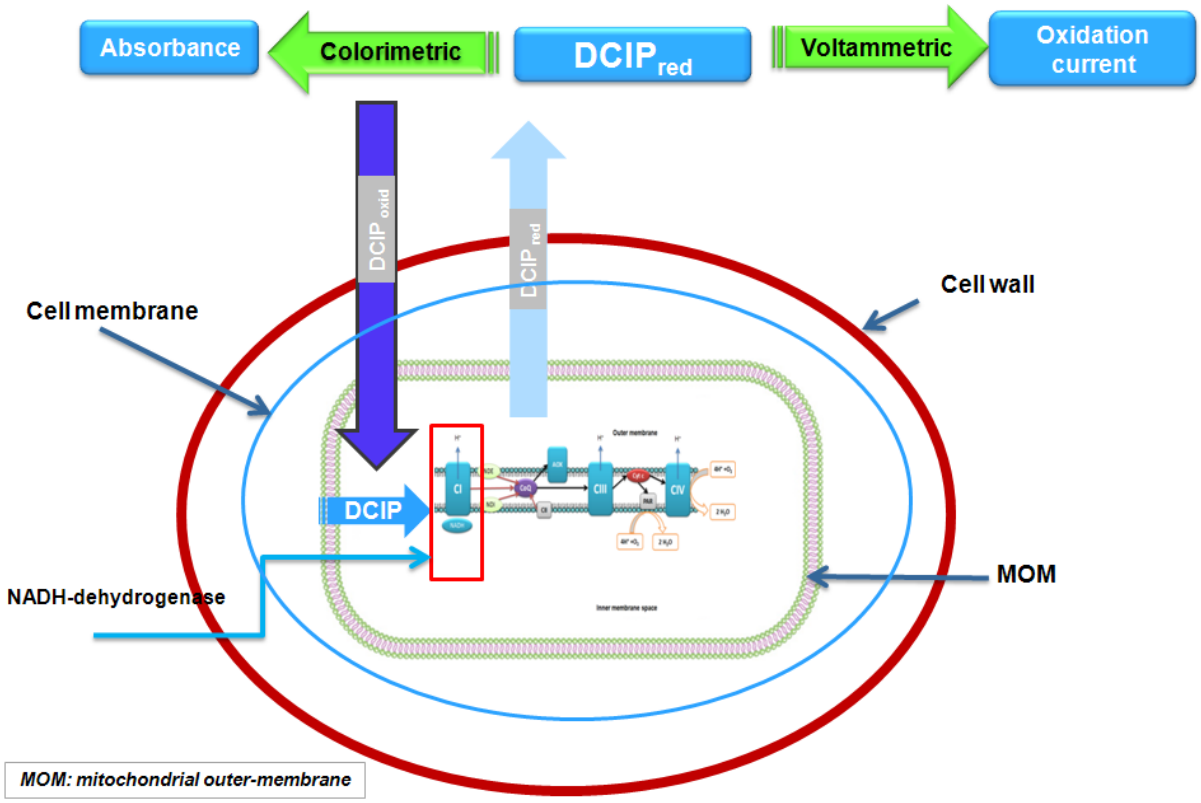
4. Discussion

In this study, we used DCIP to probe the viability of yeasts. In particular we analysed the redox activity of the complex I – positive yeast *C. albicans* and of the complex I –negative yeasts *S. cerevisiae* and *C. glabrata.* DCIP was reduced by all three yeasts and we determined the reduction of DCIP photometrically, as the reduced form of DCIP is colorless, and voltammetrically via the anodic peak current at approximately 0.6 V. Results from both detection methods correlated very well and both signals could be used as indicators for the interaction of DCIP with the yeast cells. We showed that the DCIP - reducing activity of viable cells was higher than of the chemical reduction of DCIP by NADH. In *C. albicans* it was controlled by the catalytic activity of a DCIP-NADH – dehydrogenase, which was largely rotenone-sensitive, i.e. dependent on the activity of complex I of the classical respiratory chain. Electron transport chain inhibitors of other complexes had no inhibitory effect. We also observed the catalyzed electron transfer from *S. cerevisiae* and *C. glabrata* to DCIP, but at much lower rates, and these redox reactions were rotenone - insensitive. Both yeasts lack the mitochondrial complex I, thus alternative DCIP – NADH reductases have to be present. The signals from *S. cerevisiae* and *C. glabrata* could significantly be increased by the addition of glucose, and not by the addition of galactose. Glucose is utilized in *S. cerevisiae* and *C. glabrata* (Crabtree - positive yeasts) by respiratory and fermentative pathways even in the presence of oxygen, whereas galactose is only utilized by respiration and not by fermentation. This indicated that in these yeasts respiratory pathways were not essentially involved in DCIP

reduction. The addition of either glucose as fermentable carbon source or of rotenone as inhibitor of complex I allowed the distinction between Crabtree – positive and – negative yeasts, and between complex I - positive and -negative yeasts. Moreover, in combination with other assays this differential test (+/- addition of compound of interest) could also be used for the characterization of inhibitors of the electron transport chain. In rich media inhibitors of some complexes of the respiratory chain do not affect the growth of yeasts but inhibit the consumption of oxygen. Among inhibitor candidates the DCIP test subsequently allows the identification of complex I – inhibitors, when it is performed with complex I – positive yeasts, such as *C. albicans,* without the supplementation of glucose.

5. Assay Summary

Electron transfer from microorganisms to an electrode can be achieved via electron mediators. In this study, DCIP served as a redox mediator between *C. albicans* and the working electrode. We showed that the DCIP - reducing activity could only be observed in viable *C. albicans* and was controlled by the catalytic activity of DCIP-NADH – dehydrogenase, which was largely rotenone-sensitive, i.e. dependent on the activity of complex I of the classical respiratory chain. Scheme 1 summarizes the reaction mechanism of DCIP with *C. albicans*. DCIP in the oxidized form is a lipophilic mediator that is able to penetrate the cell membranes and accepts electrons from NADH, most rapidly catalyzed by complex I of the respiratory chain. The reduced DCIP is released to the supernatant and delivers the electrons to the electrode surface generating the oxidation current, which correlates with the number of viable yeasts.



Scheme 1: Schematic representation for the interaction of DCIP with *C. albicans* and suitable detection principles.

Applications

5.1. Assessment of mitochondrial complex I activity in histidine kinase mutants

Signal transduction systems function as intracellular information-processing pathways that link external stimuli to specific adaptive responses [30, 31]. Despite the diversity in stimuli and responses, a relatively small number of molecular strategies are used for signaling. Protein phosphorylation is one such fundamental strategy. Many eukaryotic signaling cascades involve protein kinases, sensor histidine kinases (HKs), which phosphorylate both themselves and other protein substrates at specific serine, threonine or tyrosine residues, thereby regulating protein activities. In the pathogenic fungus *C. albicans*, three histidine kinases have been identified: Sln1p, Cos1p (also known as CaNik1p) and Chk1p. They are involved in the adaptation to stress, morphogenesis and sensitivity to several antifungal compounds. As HK proteins are broadly conserved and are not found in humans, it was suggested that they offer exploitation as new targets in anti-fungal drug discovery [32, 33]. Disruption of *SLN1* in *C. albicans* does not alter the viability and only slightly affects the tolerance to osmotic stress [34]. *CaNIK1* in *C. albicans* has been shown to be required for osmo- tolerance, and the deletion of this gene caused a defect in hyphal formation and reduced the efficiency of high frequency phenotypic switching [34, 35].

The histidine kinases Sln1 and CaNik1 regulate the activity of MAP kinase Hog1, which is central for stress defense reactions, whereas Chk1 seems to be related to the cell wall integrity pathways. As there were reports pointing to a connection between the HOG pathway and the respiratory chain we investigated the activity of NADH dehydrogenase in the HK deletion mutants of *C. albicans* using the DCIP assay. As shown in Figure 10 the electrical signal from Δ*sln1* reduced however Δ*chk1* and Δ*cos1* behaved as the wild-type.

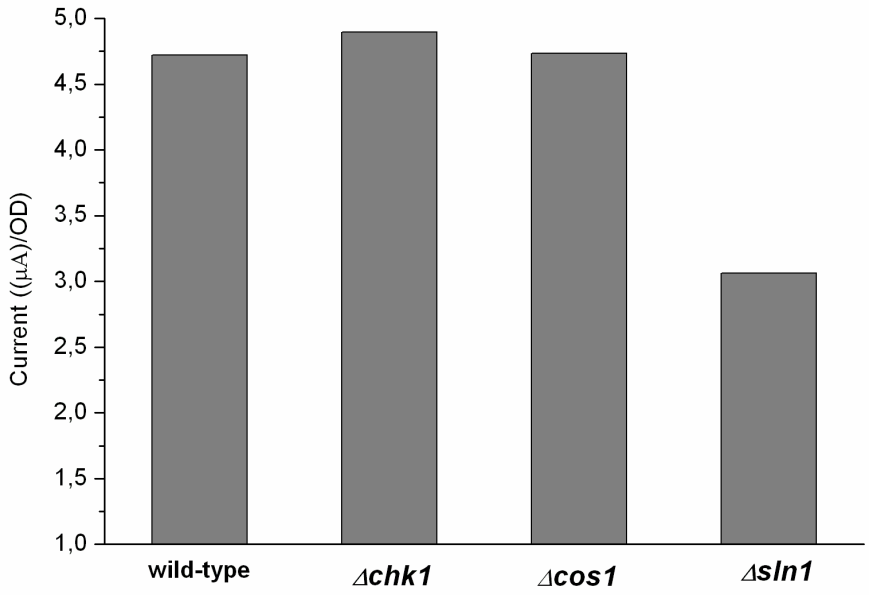


Figure 10: Voltammetric behavior of histidine kinase mutants of *C. albicans*, the peak current values represent the activity of complex I activity for each strain.

5.2. Δsln1 mutant is rotenone-insensitive

As the DCIP-assay indicated a low activity of complex I in the Δ*sln1*mutant we investigated its sensitivity to rotenone. Incubation of Δ*sln1* with rotenone did not inhibit the DCIP-reducing activity (Figure 11A) or increase the ROS production (Figure 11B). However, the wild-type was strongly affected by rotenone, whereas rotenone decreased the DCIP-reducing activity and led to increase the ROS productivity.

These results were supported by gene expression analysis (Anna Buschart, thesis 2011), which showed that the expression of several (*NAD3*, *NAD4* and *NAD5)* NADH-ubiquinone oxidoreductase subunits in addition to cytochrome oxidase subunits (*COX2* and *COX5*) were down regulated in Δ*sln1*.

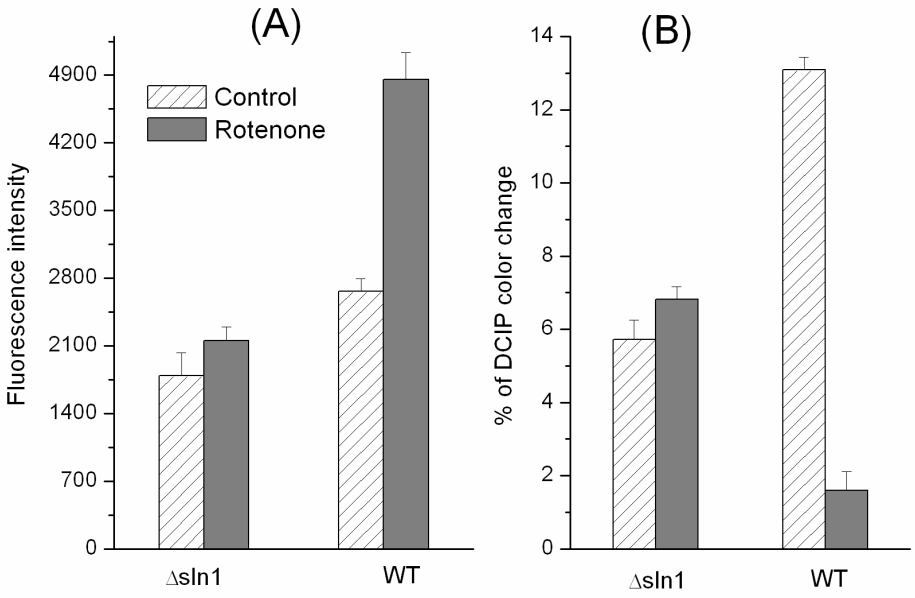


Figure 11: (A) Effects of rotenone (NADH-dehydrogenase inhibitor) on the ROS production; (B) Rotenone effect on the DCIP-reducing activity of *C. albicans* (WT) and Δ*sln1*

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Chapter 5: Summary and Discussion

Candida albicans is a polymorphic fungus that causes a range of disease in humans, from mucosal infections to systemic disease. Its ability to cause disease is linked to conversion between yeast and filamentous forms of growth.

The serious infection caused by *C. albicans*, the most common opportunistic human fungal pathogen, provides need for more rapid, sensitive antifungal treatments. The toxicity of available antifungal agents and emergence of drug resistance are major disadvantages of existing antifungal agents. Thus, finding of new drugs and biological targets for their action is required. Considering the involvement of mitochondria in many of the biological processes, for example in virulence factors of *C. albicans*, they are exploited as drug targets. For that reason, it is important to study the organization of *C. albicans* respiratory chains and determine the effect of different electron transport components on the mitochondrial functions. While in mammalian cells the organization of the classical respiratory chain is well studied and the electron transport follows a linear chain, *C. albicans* respiration involves branched respiratory pathways. Three respiratory pathways are utilized by *C. albicans* to reduce oxygen, namely the classical respiratory chain (CRC), alternative oxidative pathway (AOX) and the parallel pathway (PAR). In addition to the existence of three terminal oxidases, alternative NADH dehydrogenases (internal and external) act in parallel with complex I. These dehydrogenases catalyze the oxidation of NADH but they do not translocate protons. The complex and flexible structure of the electron transport system in *C. albicans* allows the organism to adapt to the inhibition of single complexes or pathways of the respiratory chain by the redirection of metabolic pathways or the expression of additional genes. Thus, the aim of the thesis was the establishment of a set of bioanalytical assays, the combination of which allows the elucidation of modes of action of chemical compounds and the relevance of single protein complexes. Electrochemical detection methods were of particular interest, because they offer the possibility of direct communication with the cellular electron transfer system.

A **New strategy for the evaluation of the mode of action of inhibitors of respiratory chain complexes**

C. albicans adapts to the inhibition of respiratory pathways by the expression of certain genes such as the alternative oxidase (AOX2). Thus, the analysis of effects of chemical compounds on isolated mitochondria, which is frequently used method, is not applicable to *C. albicans*. Alternative strategies are required, which make use of physiological reactions of *C. albicans* to the inhibition of various respiratory chain complexes. Thus, we set up a combination of assays, which allows to quantify different physiological parameters and from which the targeted complex can be deduced. We established this combination by the application of various specific inhibitors of electron transport chain.

In the following the results are summarized with respect to the targeted complex:

Complex I (rotenone): growth rate and viability are not affected; oxygen consumption is accelerated due to induced expression of AOX2; ROS production is increased; DCIP reduction in inhibited.

Complex II (TTFA): Inhibition of oxygen uptake; no increase of ROS production; a strong inhibition effect on the cell viability and a slight inhibition effect on the growth rate.

Complex III (antimycin; myxothiazol): A strong inhibition of oxygen uptake; a significant increase in ROS production; very strong inhibition effect on the cell viability growth rate; a strong induction of AOX2.

Complex IV (KCN): No growth inhibition; a slight effect on cell viability; increase of the oxygen uptake rate (after 25 min); decrease in ROS and very strong induction of AOX.

Alternative oxidative inhibitor (SHA): No growth inhibition effect; a slight effect on viability; inhibition of the oxygen uptake rate and a slight increase of the ROS formation

Self-mediated Electron Transfer from *Candida albicans* to Electrodes

Electron transfer processes from microorganisms to electrodes, the bio-electrochemical system, can be exploited in diagnostic tools or in microbial fuel cells. It was reported that eukaryotic microorganisms transfer electrons to electrodes without the supplement of exogenous mediators. As the origin of these processes was poorly understood, we studied the transfer of electrons from *C. albicans* and *S. cerevisiae* to carbon paste electrodes. Cyclic voltammograms (CV) showed irreversible oxidation currents in the potential range from 700 – 800 mV produced by viable yeasts. The electrical current linearly increased with increasing the cell number of viable *C. albicans*. Removal of the yeast cells from the electrolyte solution

showed that the electrochemically active compound was not bound to the cells but secreted into the electrolyte. Electroanalytical comparison of various quorum sensing compounds and mass spectrometric analysis of the electrolyte solution identified tryptophol as the electron-shuttle. This is the first report of a fungal self-mediated electron transfer process and of the function of aromatic alcohols as fungal electron mediators. The secreted amounts of tryptophol into the electrolyte solution increased with tryptophan concentration in the cultivation medium; this effect was correlated with increasing electrochemical signals. We also observed a strong involvement of the respiratory chain in tryptophol production, as the oxidation current increased with the respiratory metabolic activity of the cells. Moreover, the addition of most electron transport chain inhibitors reduced the signal, but the inhibitor of the alternative oxidase AOX, SHA, led to a significant increase of the electrical current, suggesting the involvement of the classical respiratory chain, in particular of cytochrome *c* oxidase (COX) as a relevant protein complex. The function of COX was confirmed by testing the electrochemical features of the single gene deletion mutants of COX subunits of *S. cerevisiae*. All of these mutants showed reduced electrical signals, the strongest effect being observed for the Δ*cox5a* mutant.

However, details of the regulation of the secretion of aromatic alcohols could not be elucidated with the present investigations. Usually, the productivity of alcohols, including the aromatic alcohols or fusel oils, is considered to be high when oxygen levels or the respiratory activity are low [31]. Under our conditions both the yeasts *C. albicans* and *S. cerevisiae* increased the production of tryptophol with increasing the activity of the classical respiratory chain. Moreover, it is not yet known whether only the tryptophol can act as endogenous electron transfer mediator or whether it could be replaced by tyrosol, the alcoholic product of tyrosine, when the cultivation conditions are modified. Thus, additional investigations are required in which the influence of the composition of the medium is studied and the concentrations of various alcohols are determined in different growth phases by the chromatographic analysis.

A viability assay for *Candida albicans* based on an exogenous electron transfer mediator system

In addition to the self-mediated electron transfer we studied the transfer of electrons from *C. albicans* to electrodes via 2,6 dichlorophenolindophenol (DCIP) as an exogenous redox mediator. Cyclic voltammograms (CV) showed a reversible redox reaction and an irreversible

oxidation peak only from the viable cells of *C. albicans*. The peak height of the first peak remained almost constant while the reaction time and peak shape to a current step. As the absolute current value of the second peak increased with time, but the peak form remained constant, we set a reaction time and used the peak current as measure. The oxidation peak current was proportional to the number of living cells. The reduction rate of DCIP is frequently used as indicator of the activity of complex I of the respiratory chain. However, there are also reports on DCIP-based viability assays using the complex I-negative yeast *S. cerevisiae*. Thus, we used *S. cerevisiae* together with the pathogenic complex I-negative yeast *Candida glabrata* as additional test organisms to define assay conditions, which allow a distinction of complex I-negative and positive organisms. Basal levels of DCIP oxidation were observed even with *S. cerevisiae* and with *C. glabrata*. Nevertheless, the signals were only approximately 30 % of those obtained from *C. albicans*. The carbon source glucose of galactose was added to the electrolyte solutions to activate the metabolism of yeast. The non-fermentable sugar galactose led to only a minor increase of signals from all three yeasts, whereas the addition of the fermentable sugar glucose increased the signals from *S. cerevisiae* and *C. glabrata* to the same level of *C. albicans* signals. To obtain more information about the origin of the signal we used specific electron transport chain inhibitors. As a result, only the complex I inhibitor rotenone was able to decrease the DCIP-signal in *C. albicans* whereas the other inhibitors had no effect. Thus, the DCIP-reducing activity of *C. albicans* was dependent on the complex I activity of the respiratory chain (rotenone- sensitive part). In *S. cerevisiae* and *C. glabrata* the addition of rotenone had no effect, confirming the absence of the rotenone-sensitive NADH-dehydrogenases.

The proposed assay was successfully applied to the assessment of complex I activities of histidine kinase mutants of *C. albicans*. Recent reports had indicated a link between the respiratory chains of *C. albicans* and the high-osmolarity glycerol (HOG)-pathway, which comprises the MAP-kinase Hog1 and activity of which is regulated by the histidine kinases Sln1 and Canik1. We could show that the sln1 deletion mutant had a reduced activity of NADH-DCIP-reductase, which proved to be rotenone-resistant. Thus a reduced activity of complex I in the sln1 mutant can be concluded. This conclusion is supported by the gene expression analysis showing the down regulation of genes of NADH-dehydrogenase – subunits. However, these are first results linking the function of sln1 to respiratory chain activity which requires further analysis.

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